University of Alberta

Prebiotic Treatment of Intestinal Inflammation

by

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ABSTRACT

Inflammatory bowel disease (IBD) is caused in part by a dysregulated immune response to commensal bacteria in the intestinal lumen. Probiotic bacteria are able to modulate the immune response and are able to prevent and reduce inflammation in the intestine. A relatively new approach to increase the number of probiotic bacteria in the gut is by oral administration of prebiotics. Prebiotics are non-digestible oligosaccharides that selectively stimulate growth and/or activity of a limited number of potentially health-promoting bacteria indigenous to the intestine. This study evaluated the ability of the prebiotic combination of inulin and oligofructose, Synergy1, to prevent and reduce inflammation in the HLA-B27 transgenic rat model of colitis. The second part of this study assessed if prebiotics have any in vitro anti-inflammatory properties on intestinal epithelial cells independent of probiotic bacteria. The high dose of Synergy1 prevented inflammation and a trend towards improvement in permeability was observed. There were no differences in the low dose of Synergy1 study. The cell culture work determined that there are no effects on pro-inflammatory chemokine secretion or cell viability due to prebiotic treatment. Future studies may evaluate the effect of prebiotic treatment in human IBD, and investigate the mechanisms by which prebiotics and probiotics exert their effects.

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Chapter 1: Literature Review

1.1. Introduction

Inflammatory bowel disease (IBD) is caused in part by a dysregulated immune response to commensal bacteria in the intestinal lumen. Probiotic bacteria are able to modulate the immune response in the intestine and are able to prevent and even reduce inflammation in the gut. One of the approaches to increasing the number of probiotic bacteria in the gut is to administer non-digestible oligosaccharides known as prebiotics to stimulate the growth of probiotic strains. This study was conducted to evaluate the ability of a prebiotic combination of short chain fructo-oligosaccharide (FOS) and long chain inulin to prevent the development of inflammation in a rat model of colitis. This chapter will review IBD, the intestinal immune system, intestinal permeability, the effect of probiotics, and the definition and role of prebiotics.

1.2. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) encompasses Crohn's disease (CD) and ulcerative colitis (UC), two similar yet distinct disease processes. These diseases affect the gastrointestinal system and cause the tissues to become inflamed. The etiology is believed to be a complex interaction between genetic

factors, environmental factors, and a dysregulated host immune response[1]. IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system by the normal flora present in the lumen. This improper response has been postulated to occur due to defects in the barrier function of the intestinal epithelium and the mucosal immune system[2].

Crohn's disease was described in 1932 by Burril B. Crohn, Leon Ginzburg, and Gordon D. Oppenheimer in the Journal of the American Medical Association[3]. However, they were likely not the first to observe or describe Crohn's disease, and other similar descriptions of gastrointestinal disorders have been speculated to be the same inflammatory disorder[4]. Knowledge of IBD has significantly increased since then, particularly in the past two decades. An increase in the prevalence of the disease[5], the introduction of new laboratory techniques, and the development of new animal models of IBD[6] have all contributed to improved understanding.

Patients with IBD may present with diarrhea, rectal bleeding, malnutrition and weight loss. UC only affects the mucosal layer of the colon, whereas CD inflammation is transmural and may affect any part of the gastrointestinal tract, most commonly the terminal ileum and colon. CD also has complications such as stricturing, fissures, and fistulas.

While mortality is not increased in CD and UC patients, IBD does result in substantial morbidity[1, 7]. There is no cure for IBD, and a diagnosis of IBD means lifelong management, which may include immunosuppressant and/or biological therapy and surgery. IBD also results in many direct and indirect health costs such as lost work time and increased health care costs[7-9].

1.3. Epidemiology

Epidemiologic studies conducted between 1981 and 1994 in North America list the prevalence of CD between 26-198.5 cases per 100 000 persons, and UC between 37.5-229 cases per 100 000 persons[10]. There is a slight female predominance in CD, whereas there is a slight male predominance in UC. CD is diagnosed most frequently in patients in their 20s, and UC most frequently in patients' 30s and a second peak between 50-70 years resulting in a bimodal distribution[10, 11]. However, a diagnosis of IBD can be made at any age. Ethnicity and race are long thought to be a determining factor for IBD. Historically, Caucasians and Jews have higher rates of IBD than ethnic minorities, but the distribution among ethnic groups is dynamic, and these gaps seem to be closing. Higher rates of IBD in the Jewish population, specifically Ashkenazi Jews, have been noted since the 1960s, and this holds true today. Studies in the Jewish population around the world lend some interesting insight to environmental and ethnic factors, as the prevalence is higher in Europe and North America and lower in Africa and Asia than in Israel, and the prevalence of IBD is much less

common in Israeli-Arabs[10]. These population-based studies lend themselves to the hypothesis that there are genetic as well as environmental causes contributing to IBD.

1.4. Genetics of IBD

Molecular studies based on epidemiologic findings and twin studies have led to the discovery of several susceptibility genes and genes that may determine phenotype in IBD. Some of these susceptibility loci are shared between Crohn's and ulcerative colitis, while some are specific to each form of IBD. For example, the IBD1 locus is specific to CD, IBD2 is specific to UC, and IBD3 confers susceptibility to IBD in general[12]. IBD1, found on chromosome 16, is the most characterized of IBD loci, and involves a mutation of the *NOD2* gene. The IBD5 locus on chromosome 5 contains the genes for organic cation transporters 1 and 2 (OCTN1 and OCTN2), and mutations in these genes have been linked to an increased susceptibility to CD[12]. Other associations have recently been made with ABCB1, DLG5, TLR4, TLR5, and CARD4, among others.

The best characterized gene associated with IBD is the *CARD15*/NOD2 gene found on the IBD1 locus on chromosome 16. Caspase-recruitment domain (CARD) 15 encodes for the protein NOD2, nucleotide-binding oligomerization domain 2 protein. It is composed of two N-terminal CARDs, a nucleotide binding and oligomerization domain, and ten C-terminal leucine-rich repeats (LRR) which

are thought to be involved in ligand recognition (Figure 1.1). NOD2 is a cytoplasmic protein that recognizes muramyl dipeptide (MDP), a component of the peptidoglycan found in bacterial cell walls. There are three common mutant alleles in the LRR found in patients with CD, and studies show that there is a strong gene dosage effect; the more mutant alleles present, the greater the susceptibility to developing CD. But while there is a strong association between the *CARD15* gene and developing Crohn's, it is not necessary for its development. *CARD15* mutations are found in the general population with estimates around 0.5%-2.0%, and 60-70% of CD patients lack gene mutations in *CARD15*[13]. *CARD15* mutations are absent in Japanese and Asian CD populations and in many European CD cases. Mutations in *CARD15* have been linked to earlier onset of disease, fistulizing disease, fibrostenosing disease, an increased risk for need of surgery, more familial disease, and an increase of ileal disease location[12]. The *CARD15* gene was only discovered in 2001, and research is ongoing in determining exactly how these mutations increase susceptibility to CD.

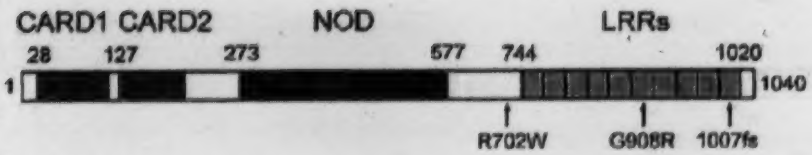

Figure 1.1. Structure of NOD2.

NOD2 has two caspase recruitment domains (CARDs) at the N-terminal end, a centrally located nucleotide-binding and oligomerization domain (NOD), and a C-terminal set of leucine-rich repeats (LRRs). The three major mutations associated with CD affect the C-terminal portion at the sites depicted by arrows.

While it is established that NOD2 acts as an MDP recognition molecule, and several signaling mechanisms have been identified, the physiological functions are less well understood. One hypothesis is that the NOD2 mutation may cause impaired epithelial defense against bacteria, possibly allowing an overgrowth of bacteria or a shift towards more inflammation-inducing bacteria, thus inducing an inflammatory response.

Great strides have been made in understanding the genetic contribution to IBD. Several loci have been shown to increase susceptibility and influence disease phenotype. It is hoped that with a more complete understanding of IBD genes, prevention and treatment strategies may even be tailored to the genetic type of IBD.

1.5. Intestinal Immunity

Current evidence lends itself to the hypothesis that CD and UC may be a result of dysregulated immune function. The gastrointestinal system has by far the greatest antigenic and bacterial load of any system in the body. This leads to extensive immune stimulation and activation of mononuclear cells in the normal mucosa. This process is tightly regulated and is known as "physiological" or "controlled" inflammation. Any inappropriate changes in maintaining this careful balance between inflammation and tolerance can lead to "uncontrolled inflammation" which can be damaging to the surrounding tissue as is seen in the two major forms of IBD[14].

Under physiological conditions, the immune system samples the contents of the lumen with the epithelial cells, specialized M cells, and with dendritic cells, whose projections can span the epithelial layer into the lumen without disrupting tight junctions. These antigen-presenting cells (APCs) then activate the immune system by presenting luminal antigens to subsets of T lymphocytes. T-helper type 1 cells (T_h1 cells) secrete interferon-gamma (IFN-γ) as well as tumor necrosis factor-alpha (TNF-α). Dendritic cells and macrophages activate the T_h1 cytokine response usually when triggered by an invading pathogen by secreting interleukin-12 (IL-12). T_h2 cells are characterized by their secretion of IL-4 and IL-10 which drive the production of immunoglobulin A (IgA). The T_h2 cytokine response is usually activated during parasitic infection, such as by helminthes. T-regulatory (T_{Reg}) cells / T_h3 cells secrete IL-10 and transforming growth factor-

beta (TGF-β) which induces B cells to switch from the IgM to the IgA isotype and maintain oral tolerance. In healthy individuals, inflammation is controlled by T_{Reg}/T_h3 cells which limit the inflammatory response, and by apoptosis of T cells once the pathogen has been dealt with.

1.5.1. Crohn's Disease

CD is characterized by a T-helper type 1 (T_h1)-mediated immune response. Macrophages and dendritic cells secrete IL-12 in response to exposure to bacterial products. One of the most powerful and relevant activities of IL-12 is its ability to shift the immune response in a T_h1 direction[11, 15]. This response is characterized by increased production of interferon-gamma (IFN-γ), IL-1β, IL-2 and IL-6, which results in a positive-feedback loop sustaining activation. IL-18, another T_h1-associated cytokine, is secreted by intestinal epithelial cells and lamina propria mononuclear cells (LPMC) and acts synergistically with IL-12 to activate T_h1 cells[15]. The result of T_h1 activation is an increase of chemokine secretion, activation of macrophages and lymphocytes[16], inhibition of differentiation of T_h0 cells to the T_h2 cell type, alteration of tight junctions between adjacent intestinal epithelial cells[17], activation of granulocytes, recruitment of neutrophils[18], and activation of matrix metalloproteinases[19]. There appears to be a lack of control of inflammation in CD. Deficiencies in T_{reg} cells have been observed. In addition, mucosal T cells are resistant to apoptosis, leading to

accumulation of T cells and perpetuation of the immune response[19]. Together, these effects perpetuate the inflammatory response and further tissue damage.

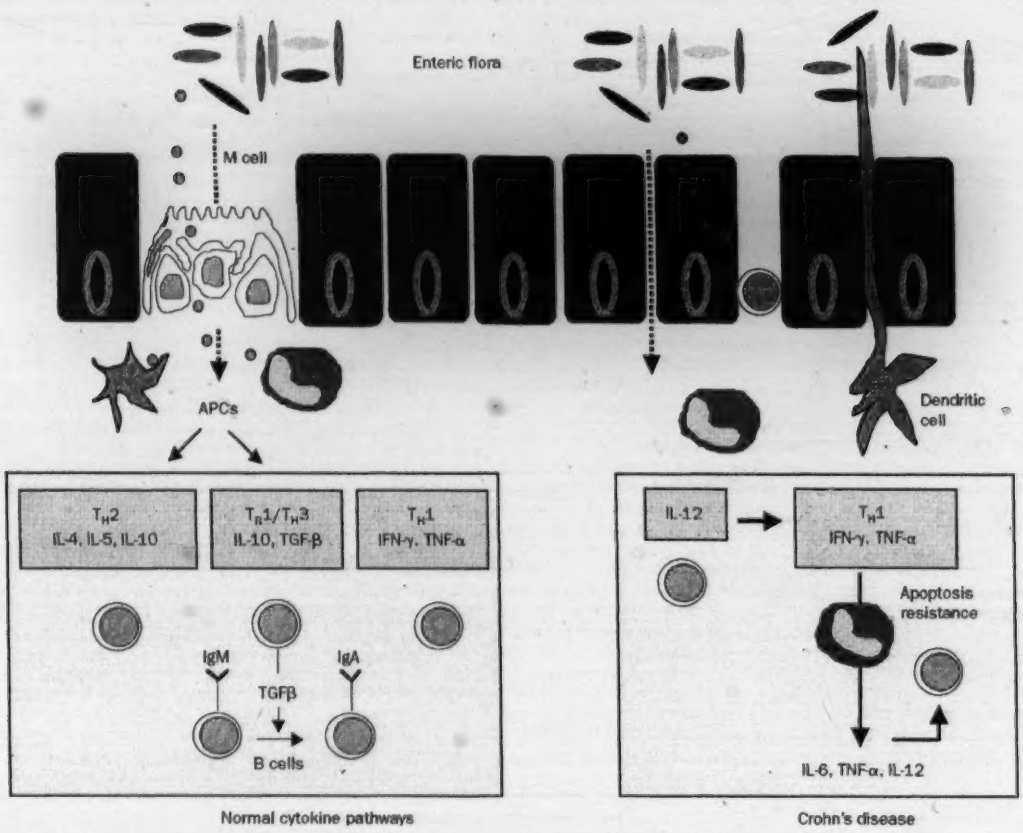

Figure 1.2. Cytokine balance within the healthy mucosa and in Crohn's disease

Antigens within the enteric microenvironment are sampled by uptake across specialized epithelial M cells and presentation to the immune system by antigen-presenting cells (APCs) which induce dendritic cells and macrophages. The columnar epithelium also serves sensory function and subepithelial dendritic cells could sample the lumen by sending processes across the epithelium. Healthy mucosal cytokine responses vary between species and are affected by genetic and environmental factors, including the composition of flora. In human beings, both T_h1 and T_h2 cytokine pathways exist with regulatory cytokines IL-10 (by T_{Reg} cells) and transforming growth factor β (TGF-β) and T_h3. In Crohn's disease, APCs generate mainly IL-12, thereby driving a T_h1 response. Resistance of T cells to apoptosis is due to trans-signaling by IL-6 and contributes to accumulation of activated T cells and chronicity of disease. IFN=interferon, TNF=tumor necrosis factor.

(From Shanahan F. Crohn's Disease. The Lancet 2002.[19])

1.5.2. Ulcerative Colitis

The immune cell and cytokine profile for ulcerative colitis is less clear. Clinical and histopathological presentation is very different from CD. The damage is limited to the mucosa, which is abounding with neutrophils and severe epithelial damage, contrasting the transmural inflammation of CD. At present, a T_h2-mediated humoral response is hypothesized. T_h2 cells produce IL-4, IL-5, IL-10 and IL-13, and specialized natural killer T cells (NKT cells) also produce IL-13. Data to support this hypothesis includes the finding that lamina propria T cells (LPMT) from UC patients produce significantly higher levels of IL-13 and IL-5, and little IFN-γ when compared with controls. How this actually leads to the cascade of inflammatory events has yet to be determined though. The T_h2 paradigm may have some merit in that it may participate in antibody-mediated autoimmunity. In UC, the inflamed colon is filled with IgG plasma cells, and IgG colocalizing with complement is visible on the surface of epithelial cells. The IgG response is likely T-cell dependent and generated in gut associated lymphoid tissue (GALT)[15]. Further evidence to the autoimmunity hypothesis includes the fact that approximately 70% of UC patients express anti-neutrophil cytoplasmic antibodies with a perinuclear staining pattern (pANCA). This auto-antibody may be present due to cross-reactivity with commensal bacteria. More evidence lending to the auto-antibody theory includes the fact that UC is organ specific and can be "cured" with a colectomy. There is also evidence to a role for peroxisome proliferator-activated receptor-γ (PPAR-γ), which is thought to inhibit colitis through attenuation of nuclear factor κB (NFκB) activity. Results show that

PPAR-γ expression is reduced in UC compared to healthy controls and CD[15, 20]. The pathogenesis of UC appears to be driven by bacterial antigens and products which lead to dysregulated immune function. Differences in the underlying mechanisms may reflect different phenotypes and ultimate causes UC.

1.6. Intestinal Barrier

The intestine is charged with the daunting task of preventing invasion by microorganisms, while avoiding a response to antigenic stimuli from commensal bacteria and food proteins and allowing for the uptake of nutrients. It accomplishes this by forming a physical barrier of epithelial cells joined together by tight junctions and by secreting a chemical barrier.

1.6.1. Physiochemical Barrier

The physiochemical barrier is the first line of defense against bacteria and antigens in the lumen of the gut. It includes chemical barriers like acid-base challenges, bile salts, and brush border enzymes. It also includes the mucous layer that covers the surface of the epithelial cells. Mucous is composed of a small protein core and a complex array of oligosaccharide chains. They act as a selective physical barrier for some macromolecules and microorganisms, it allows for accumulation of bacteriocidal and bacteriostatic chemicals and enzymes, and it holds immunoglobulin A (IgA), which acts to reduce the antigen load. Mucous

also prevents mechanical damage of the mucosa. If the mucous layer is absent, inflammatory responses to noxious stimuli are enhanced[21, 22].

1.6.2. Epithelial Barrier

One of the mechanisms by which the intestine prevents invasion by microorganisms is by a single layer of epithelial cells and proteins connecting adjacent epithelial cells. There are three components to the barrier formed between cells: the tight junction (or zonal occludins) closest to the luminal surface, the zonal adherens or adherens junction (AJ), and the macula adherens most basolaterally. Paracellular permeability across the epithelium is regulated primarily by the tight junctions (TJs) that form a circumferential band around the apical poles and connect adjacent cells. The TJ acts as a boundary between the apical and basolateral plasma membranes which contain different lipids and proteins and have different functions, and it helps to maintain cell polarity. The TJ is comprised of a complex of proteins and lipids that are linked to the underlying actin cytoskeleton and extend into the intercellular space between adjacent epithelial cells. These affiliations with the perijunctional actin ring play an important role in regulation of the TJ in physiological and pathological states. The fibrils between adjacent cells consist of transmembrane proteins occludin ("to occlude"), junction adhesion molecule (JAM), and members of the claudin family ("to close"). JAM is a member of the immunoglobulin superfamily. Its C-terminal is intercellular, it spans the membrane once, and forms two Ig-like domains extracellularly[23]. Occludin and the claudins are transmembrane proteins that

span the membrane four times forming two extracellular loops that interact homo- and heterotypically with similar loops from adjacent cells. Intracellularly, the C-terminus is linked to the actin microfilaments by a number of cytoplasmic proteins including the zonal occludins proteins (ZO)[24]. ZO-1, an underlying scaffolding protein, links transmembrane proteins to ZO-2, ZO-3 and actin.

1.6.3. Paracellular Permeability

Paracellular permeability is determined by the integrity of the TJ complex. It is regulated by such things as the number and types of TJ subunits and components[25], by the concentration of meal-related nutrients (ie. increased glucose increases permeability)[26], bacterial invasion (ie. enteropathogenic *E. coli* increases permeability)[27] and by cytokines. Functional alterations in the cytoskeleton help regulate permeability; phosphorylation of myosin light chain by myosin light chain kinase (MLCK) leads to perijunctional actinomyosin ring contraction, increased tension on the tight junction, and hence regulated increases in TJ permeability[27].

1.6.4. Epithelial Barrier and IBD

An increase in permeability is postulated to be a primary defect contributing the etiology of IBD and can help predict relapse in CD patients[28]. An increase in permeability can also be measured in first degree relatives of CD patients[29] as

well as in spouses of CD patients[30], lending support to both the genetic and environmental hypotheses for the causes of IBD.

Several studies have been undertaken to determine the relationship between tight junction proteins and IBD. Kucharzik et al. studied TJ and AJ proteins in colorectal epithelial biopsies from IBD patients and found that occludin, claudin-1, JAM, and ZO-1, E-cadherin and β-catenin were all down-regulated in areas adjacent to neutrophils. Occludin expression was diminished in epithelials away from neutrophils[31]. Researchers are now investigating why these barrier proteins are downregulated and the mechanism by which this is occurring.

CD is characterized by a T-helper type 1 cytokine profile and proinflammatory cytokines such as TNF-α and IFN-γ have been measured in the intestinal tissues of CD patients[32]. IFN-γ is released by activated T cells and by natural killer T cells. Increased secretion of IL-12 and IFN-γ leads to macrophage and granulocyte activation, leading to increased release of IL-6 and TNF-α[33].

Studies on intestinal epithelial TJs show that IFN-γ increases permeability by disassembly of the TJ complex, however this is not an acute phase response; one study documents it commencing at 38 hours after treatment and reaching a maximum at 48 hours after IFN-γ treatment of T84 monolayers. The mechanism determined is that IFN-γ stimulates macropinocytosis of TJ proteins (rather than

clathrin-mediated or caveolar-mediated means of endocytosis). Bruewer et al. were able to show that occludin, claudin-1, and JAM-A, were internalized to early/recycling endosomes[17]. The reasons as to why IFN-γ-induced internalization of the TJ proteins is delayed have yet to be determined, but it is hypothesized that it could be due to an upregulation of proteins involved in intracellular vesicle trafficking.

TNF-α also increases permeability, although its mechanism is quite different from that of IFN-γ. TNF-α increases MLCK protein expression. Ye et al. show that this is accomplished through an increase in MLCK promoter activity mediated by IκB degradation, NF-κB translocation to the nucleus, and NF-κB binding to the downstream κB binding site on the promoter. The increase in MLCK results in increased contraction of the actomyosin ring[34]. Gitter et al. also determined that TNF-α induces apoptosis of intestinal epithelial cells, contributing to the leakiness of the barrier[35]. TNF-α-antibody therapy was able to restore epithelial apoptosis to normal levels in CD patients, as analyzed by sigmoid colon biopsies before and 14 days after treatment[36].

UC is characterized by a T-helper type 2 response and by an increase in permeability. The increase in permeability is due to epithelial cell apoptosis and a decreased complexity of the TJs. The T_h2 response is associated with an increase in IL-13. IL-13 is released by natural killer T cells (NKT cells). Such

NKT cells can be found in the lamina propria and peripheral blood of UC patients. Lamina propria mononuclear cells (LPMC) isolated from UC patients produced significantly higher amounts of IL-13 compared to samples from CD patients and controls. IL-13 has been shown to induce apoptosis of intestinal epithelial cells, impair epithelial restitution (migration of neighbouring cells into gaps in the epithelial monolayer), and increase expression of claudin 2 (CL2). CL2 is a TJ protein which increases paracellular permeability and is known to have increased expression in UC[37]. The effects elicited by IL-13 are enhanced by TNF-α, which is elevated in macrophages in UC. TNF-α improves IL-13-induced STAT-6 activation thus enhancing the downstream effects of IL-13. The synergism between IL-13 and TNF-α may explain why some UC patients respond to anti-TNF-α antibody treatment[33].

1.7. Intestinal Microflora

The human intestine is estimated to contain 10^{14} bacteria composed of approximately 400 species[38-40]. Colonization of the intestine with bacteria begins at birth when the infant passes through the birth canal. While genetics of the host and environmental factors may modulate the distribution of the flora, diet appears to be a factor that has the greatest impact on the concentration of species present in the gastrointestinal tract[39].

The number and type of microorganisms inhabiting the different sections of the intestine are determined by intrinsic (host-mediated) factors, extrinsic factors, as well as by the microbes themselves. Many express adhesion factors that allow them to take up residence in a particular part of the GIT. In return for providing a suitable environment in which to live, the bacteria provide the host with many benefits such as degrading some food components, production of certain B vitamins, production of short chain fatty acids (providing an estimated 40-50% of the energy requirement for colonocytes), and producing digestive and protective enzymes[38]. Probiotic bacteria have additional benefits such as modulating the host immune response, enhancing epithelial barrier function, and antagonizing pathogenic bacteria[39].

1.7.1 Probiotics

Probiotics are living microorganisms that affect the host in a beneficial manner by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract[39]. The benefits of probiotics have long been known. In a Persian version of the Old Testament (Genesis 18:8) it states that "Abraham owed his longevity to the consumption of sour milk." In 76 AD the Roman historian Plinius recommended the administration of fermented milk products for treating gastroenteritis[41]. In the early 20th century, Elie Metchnikoff claimed that the intake of fermented milk products could favourably shift the intestinal bacterial balance, reduce the toxin-producing bacteria in the gut, and improve longevity[41]. The term "probiotic" was first applied by Lilly and Stillwell in

1965 to describe "substances secreted by one microorganism which stimulate the growth of another", and thus was contrasted with the term "antibiotic".

Today, probiotic bacteria are touted as having many beneficial health effects such as improving intestinal mucosal barrier function, modulating intestinal microflora, and modulating immune function, thus preventing and even treating some intestinal and extra-intestinal diseases[42]. They may do this by modulating the host's immune function, providing competition for pathogens, and by improving nutritional balance in the intestinal tract[39, 43]. Probiotic bacteria exert their effects through a variety of mechanisms unique to each strain (Box 1). The two main genera associated with having probiotic activity are *Lactobacillus* and *Bifidobacteria* though there are several other microorganisms that have been used as probiotics, including but not limited to other lactic acid bacteria (LAB) strains (Table 1).

Box 1. Biological effects of probiotic bacteria

Modulation of host immune response
- Modulation of NF-kB and AP-1 pathways
- Altered cytokine release
- Induction of regulatory T cells
- Induction of PPAR-γ

Enhanced epithelial barrier function
- Enhanced tight junction protein phosphorylation
- Upregulation of mucous production

Anti-microbial effects/antagonism against pathogens
- Decreased luminal pH
- Secretion of anti-microbial peptides
- Inhibition of pathogenic bacterial invasion
- Blockade of bacterial adhesion to epithelial cells
- Release of nitric oxide

Modified from Penner R, and Salminen S[39, 44]

Table 1.1. Microorganisms in probiotic products

Lactobacillus species	Bifidobacterium species	Other LAB	Non-lactics
L. acidophilus	*B. adolescentis*	*Ent. faecalis*	*Bacillus cereus ('toyoi)*
L. casei	*B. animalis*	*Ent. Faecium*	*Escherichia coli* (Nissle 1917)
L. crispanis	*B. bifidum*	*Lactoc. lactis*	
L. gallinarum	*B. breve*	*Leuc. mesenteroides*	*Propionibacterium freudenreichii)*
L. gasseri	*B. infantis*	*Ped. acidilactici*	
L. johnsonii	*B. lactis*	*Sporolactobacillus inulinus*	*Saccharomyces cerevisiae ('boulardii)*
(L. paracasei)	*B. longum*	*Strep. thermophilus*	
L. reuteri			
L. rhamnosus			

Reference: [38]

1.7.2. Utilization of Carbon Sources

Probiotic bacteria are part of a larger family of bacteria known as lactic acid bacteria (LAB). Lactic acid bacteria (LAB) are "Gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates" [45]. LAB ferment hexoses such as glucose, mannose, galactose, and fructose, as well as pentoses. Larger sugars such as lactose, maltose, and sucrose are cleaved into monomers which can then enter the fermentative pathways. Several LAB are also able to use larger carbohydrates such as prebiotics.

1.7.3. Prebiotics

Prebiotics are non-digestible oligosaccharides that selectively stimulate growth and/or activity of a limited number of potentially health-promoting bacteria, including bifidobacteria indigenous to the intestine[46]. Oligosaccharides differ from one another in the number (2-60) and type of hexose moieties they possess (ie. glucosyl-, fructosyl-, galactosyl-, xylosyl-), and the linkages (β and α) between the hexose moieties. These characteristics have consequences on their fates in the gastrointestinal tract[47]. Some of these oligosaccharides and their sources have been summarized in Table 3. Their chemical structure makes them relatively resistant to digestion by hydrolytic enzymes active in the human intestine (ie. α-glucosidase, maltase, isomaltase, and sucrase). Inulins (fructo-oligosaccharides with linear β(2→1) frutosyl-fructose linkages are completely resistant, β(2→6) fructo-oligosaccharides may be partially hydrolyzed at low pH, and isomalto-oligosaccharides have good resistance when the degree of polymerization is greater than three[47]. Bacteria residing in the cecal and colonic segments of the digestive tract may have enzymes such as β−fructosidase, β−galatosidase, α-galactosidase, xylanase and other hydrolases that are able to break these carbohydrates down[47]. The effects prebiotics have on the intestinal microflora, and therefore host health, depend upon which bacteria are able to use the prebiotic substances, what they do with them, and how the affected bacteria interact with intestinal functions [48].

Table 1.2 Prebiotic oligosaccharide structure and sources

Starch	Structure	DP	Linkage	Examples	Sources
Lactulose	Galβ1-4Fru		β (1→4)	Cephulac®	Synthesis from Lactose
Fructo-oligosaccharides (inulin, FOS)	Gluα1→2βFru1→2]n	2-60	β (1→2)	Raftilose (DP 2-9) Raftiline (DP >10)	Natural: Fruits and vegetables, onions, banana, garlic. Industrial: Synthesis from saccharose, hydrolysis from chicory root inulin
Xylo-oligosaccharides (XOS)	Xylβ1-4[Xyl]$_n$	2-7	β (1→4)		Natural: plant-derived Industrial: Hydrolysis from polyxylans
Soybean oligosaccharides (SOS)	Raffinose: Galα1→6Gluα1→2βFru Stachyose: Galα1→6Galα1→ 6Gluα1→2βFru		α(1→6)	Raffinose Stachyose	Soyabeans
Isomalto-oligosaccharides (IMO)	Gluα1→6[Gluα1→6]n	>1 (avg 1-2)	α1→6) or α1→4)		Hydrolysis and glycosyl transfer from starch
Galacto-oligosaccharides (GOS)	α-Glu1→ 4[αGal1→6]n	1-4	β (1→4)	Oligomate 55	Natural: Human milk Industrial: Enzymatic synthesis from lactose
Levan	β-D-Fru-(2→6)-(β-D-Fru)$_n$	6-19	β2→6 β2→1		Synthesized by levanase - containing bacteria (ie. L. reuteri [49] and Erwinia spp.[50])

Adapted from references [47, 51, 52]

Inulin and oligofructose (FOS) are among the most studied and well-established prebiotics. These prebiotics have been shown to have a bifidogenic effect in a rat model of colitis[53] and humans[54]. Several other studies demonstrate selective fermentation of prebiotics by beneficial flora, in particular bifidobacteria and to a lesser extent lactobacilli[55].

Because probiotic bifidobacteria are susceptible to oxygen and heat making their application in foods and supplements difficult, there is an increasing interest in the bifidogenic properties of food-grade prebiotics such as FOS and inulin. Their ability to increase the number of probiotic strains of bacteria in the gut have made them an attractive focus for not only promoting normal digestive health, but also for preventing and treating digestive diseases such as IBD.

1.8. Summary

The gastrointestinal tract is exposed to the greatest bacterial load of any system in the body, and one of its fundamental roles is to prevent invasion by pathogenic bacteria while also preventing inappropriate immune activation by commensal bacteria and antigens. The intestinal barrier prevents entry of luminal contents into the body while the intestinal immune system samples these luminal contents for potentially harmful components. The gastrointestinal system also promotes its own health by providing a favourable environment for bacteria, especially probiotic bacteria. Research has only just begun to understand the complex

interaction between the human host and the bacteria living within, and will continue to be an exciting and insightful topic of research.

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Chapter 2: Models for Study

2.1. *in vivo* Studies: HLA-B27 Transgenic Rats

HLA-B27 transgenic rats express human leukocyte antigen B27 (*HLA-B27*) and human *β2-microglobulin* (*β2m*) genes and exhibit a phenotype similar to humans suffering B27-related rheumatic disorders, including inflammatory bowel disease[1].

HLA class I and class II molecules are peptide-presenting molecules for T-cells. T cells are unable to recognize antigens directly, and can only recognize short segments of peptide bound to major histocompatibility complex (MHC) molecules, the human form of this being HLA molecules. The HLA B complex belongs to class I and consists of two chains: the α or heavy chain which spans the cell membrane, and the β2-microglobulin chain. The α and β chains are associated non-covalently. While the β2-microglobulin (β2m) chain is invariant, the α chain is highly polymorphic. The α chain contains three domains, and the peptide binding occurs between the α1 and α2 domains. The set of anchor residues that bind the peptide in the pocket of the two α chains is called a sequence motif[2]. The MHC-bound peptide can then be presented to $CD8^+$ T lymphocytes.

The HLA-B27 β2m complex is implicated in the susceptibility to the chronic inflammatory disease ankylosing spondylitis (AS) and related spondyloarthropathies. While genetic susceptibility to AS is complex, the role of HLA-B27 is clear: 95% of Caucasians with AS carry the gene while only 7-8% of healthy controls carry it [3]. It has yet to be elucidated why the HLA-B27 complex leads to inflammation. It has been proposed that the HLA-B27 protein has a tendency to misfold in the endoplasmic reticulum (ER), possibly due to three pocket residues virtually unique to the protein. This can disrupt homeostasis and activate signal transduction pathways collectively known as the unfolded protein response (UPR) that ensure the protein folding capacity of the ER is not overwhelmed. ER stress and UPR activation have pathological consequences and may disrupt immune function when they occur in antigen presenting cells (APC) such as macrophages and dendritic cells[3]. This is likely not the only mechanism by which the HLA-B27 complex leads to immune dysfunction, and much work remains in clarifying exactly why it leads to spondyloarthropathies.

Intestinal inflammation in AS can resemble that of IBD. Clinically overt inflammation of the gut has been reported to be present in 5-10% of AS patients, subclinical gut inflammation as detected with ileocolonoscopy has been found in 25-49% of AS patients, and microscopic lesions detected with histological analysis of gut biopsies are reported as high as 50-60% of AS patients. Increased intestinal permeability has been observed in patients with AS similar to what is seen in IBD patients. In addition, increased gut permeability has been

measured in relatives of patients with IBD as well as relatives of AS patients[4]. Other similarities between AS and IBD have been drawn with respect to immunopathology and clinical response to pharmaceuticals such as infliximab. Resemblances between AS and IBD have led to using animal models of AS in the study of IBD.

The spontaneously arising disease in HLA-B27/hβ2m transgenic (HLA-B27 TG) rats has a striking clinical and histological resemblance to B27-associated disease in humans[1]. This model was first developed to study ankylosing spondylitis, but in light of the similarities between AS and IBD, it was soon adapted as a model of IBD. Both IBD and the HLA-B27 model appear to require bacteria for disease initiation and progression. Evidence towards this includes the observation that the most prevalent site of inflammation in the transgenic rats is in the gastrointestinal tract, which suggests that events which initiate the disease process occur in the GI tract[1]. Also supporting this hypothesis is the fact that germ-free HLA-B27 transgenic rats do not develop colitis[5]. The HLA-B27 rat is similar to IBD in that bacterial recognition genes are implicated in their pathogenesis. In IBD, polymorphisms in *NOD2* and *TLR4*, genes associated with bacterial recognition, are associated with an increased risk of developing IBD[6]. Similar studies in the HLA-B27 model and humans further lend evidence to their similarities. When the fecal stream is diverted, inflammation is attenuated in both IBD and the HLA-B27 model[7; 8]. Antibiotics prevent disease in the HLA-B27 model[9], and they've been shown to improve disease scores in IBD[8]. Increases in

E. coli and *Bacteroides* species and decreases in *Lactobacillus* and *Bifidobacteria* species have been observed in IBD patients in comparison to healthy controls[8]. In the HLA-B27 model, *B. vulgatis* has been determined to be a major factor contributing to inflammation[10, 11], and increases in *Lactobacillus* are associated with a decrease in inflammation in this model[12, 13]. The similarities in the bacterial role of the pathogenesis of the inflammatory process make the HLA-B27 a useful model for IBD.

While the functional characteristics of the HLA-B27 transgenic rat model may mimic IBD, it is not an entirely perfect model. It approximates the general characteristics of IBD, but certain aspects are inconsistent with each of the two major forms of IBD. While HLA-B27 TG rats develop inflammation histologically resembling UC[14], the cytokine profile resembles that of CD and suggests a prominent T_h1 response[15]. The genetic basis for disease in the HLA-B27 TG rat also differs from IBD. Genetic susceptibility for IBD includes at least seven confirmed loci and as many as eight more potential loci[16]. The inflammation arising in the HLA-B27 model does not occur due to these genetic susceptibility factors. However, the inflammation that occurs in this model resembles IBD well enough to be used for gaining new insights into the mechanisms of disease and exploring new treatments for IBD.

2.2. *in vitro* Studies: HT-29 Cells

Intestinal epithelial cells are in continuous contact with the intestinal microflora and luminal contents. They participate in mucosal defense by preventing entry of pathogens, secreting protective factors, acting as antigen-presenting cells, and expressing cytokines and chemokines in response to pro-inflammatory cytokines and luminal antigens[17]. A number of transformed colonic cells lines have been developed for studying interactions with colonic cells and intracellular mechanisms. One of these cell lines is the HT-29 cell line.

The HT-29 cell line was established by Jorgen Fogh in 1964 from a human colon carcinoma. Under standard culture conditions (ie. in the presence of glucose and serum), HT-29 cells grow as a multilayer of unpolarized undifferentiated cells. Depending on media composition, these cells can be differentiated to functionally resemble certain intestinal cell types[18]. The cells used in this study were undifferentiated, but were grown in polarized monolayers by culturing them in plates that promote this type of growth.

HT-29 cells secrete several cytokines and chemokines in response to pro-inflammatory cytokines and bacterial invasion signals. Eckmann et al. showed that they would secrete IL-8 in response to TNF-α, IL-1β, IFN-γ and lipopolysaccharide (LPS)[19]. TNF-α is released by macrophages during an inflammatory response and can be used to mimic inflammation in HT-29 cell

culture. Jung et al. demonstrated that HT-29 cells have markedly increased mRNA levels and increased secretion of several pro-inflammatory mediators including IL-8 in response to TNF-α[20]. IL-8 acts as a potent chemoattractant and activator of neutrophils, which are involved in inflammation in IBD. Elevated IL-8 correlates with an increased inflammatory response.

The HT-29 cell line is useful for studying IBD. They are derived from human colonocytes, and so are useful in studying human conditions. They are well characterized, verified, and a widely accepted model of human intestinal cells. HT-29 cells are a homogeneous cell preparation, have long-term viability, and respond strongly and predictably to stimulation by inflammatory mediators. They express the same array of cytokines and chemokines in response to bacterial invasion as they do to pro-inflammatory mediators such as TNF-α, making it easy to standardize stimulatory signals for study. In contrast, freshly isolated human colonic cells are heterogeneous, have limited viability, and cytokine expression varies sample to sample.

HT-29 cells have limitations however. They are not normal cells; they are a malignant transformed cell line. There are differences between this cell line and freshly isolated colonic cells. For example, freshly isolated cells will express IL-6, whereas HT-29 cells do not[20]. They differ slightly in function compared to colonic cells, and they do not form tight junctions between cells[18]. Another cell line must

be utilized for studying resistance and permeability. Despite their limitations, HT-29 cells provide a useful model for studying cytokine expression.

2.3. Two methods for solving one problem

There are many advantages to employing both *in vivo* as well as *in vitro* studies when approaching a biological problem. *In vivo* methods most closely resemble the actual conditions of a biological system, which can be very complex. *In vitro* methods are reductionist in nature and are used to minimalize the number interactions occurring to isolate the effect of a treatment on one particular cell type.

In vivo studies look at the broad picture in a biological problem. General interactions between different cell types and body systems, as well as bacteria-host interactions can be studied. The processes that occur in an actual human, in an actual disease state are replicated as closely as possible, and data on the effects on the entire body is collected. However, *in vivo* studies have limitations. While *in vivo* studies are very good for looking at the overall effect of interactions mentioned above, they are limited in their ability to determine the specific interactions and processes.

In vitro studies are very useful for clarifying specific interactions and cellular processes that occur in an *in vivo* system. The type of cells and number of

interactions that can occur are reduced. In doing so, the effects of a particular treatment on a particular cell type under specified conditions can be determined. The strengths of the reductionist model can also be a limitation though. The consequences of a treatment on a particular cell type may not reflect what occurs under *in vivo* conditions; effects of a particular treatment may depend on interactions between other cells, or may even depend on interactions with the environment that are not or cannot be replicated in an *in vitro* system. Utilizing both whole-animal and reductionist models in a study clarify how a treatment is exerting its effects and what the wider-reaching consequences are.

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Chapter 3: *in vivo* studies

3.1. Overview

The intestinal microflora is implicated in the pathogenesis of IBD in humans, as well as in the pathogenesis of intestinal inflammation in the HLA-B27 transgenic rat model of colitis. Probiotics have proven effective in reducing inflammation in both IBD and in the HLA-B27 model[1, 2]. Two mechanisms by which probiotics likely exert their beneficial effects include immune modulation and enhancement of intestinal barrier function[2]. Prebiotics are non-digestible oligosaccharides that have been shown to modify the microflora of the intestine and increase numbers of probiotic bacteria such as *Bifidobacterium* and *Lactobacillus*. The aim of this study was to investigate the effects of prebiotics on intestinal inflammation and permeability in the HLA-B27 TG model of colitis.

3.2. Methods

3.2.1. Animals

Male HLA-B27 transgenic rats (the 33-3 line on an F344 background) and their non-transgenic littermates were obtained from the colony in the Health Science Laboratory Animal Services (HSLAS) division within the Faculty of Medicine and

Dentistry and were housed in specific pathogen-free (SPF) conditions in the facility in the Dentistry/Pharmacy Building. Animals were originally purchased from Taconic. All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and with the permission of the University of Alberta Health Sciences Animal Policy and Welfare Committee (protocol 423-01-05). Presence or absence of the HLA-B27 transgene was determined by PCR using DNA isolated from tail clippings. Rats were housed in temperature and humidity controlled rooms with a 12 hour light-dark cycle. After weaning, rats were housed in groups of one to three rats/cage. Rats were fed a standard rat chow (5001 Rodent Diet: Lab Diet, Brentwood MO, USA). All animals had access to food and water *ad libitum* throughout the study.

3.2.2. Prebiotic Treatment

Intestinal inflammation and increased intestinal permeability in the HLA-B27 TG rat develops over the first 8-16 weeks of life[3-6]. To assess the ability of the treatment to prevent inflammation, the rats began treatment prior to the development of inflammation and permeability defects. At four weeks of age, rats were randomly assigned to one of two groups. The first group received a combination of chicory derived long-chain inulin and short-chain inulin fraction oligofructose in a mixture of 1:1 (Raftilose Synergy, Orafti, Tienen, Belgium) in autoclaved drinking water at a dose of 7.5g/kg bodyweight per day. (This prebiotic mixture will be referred to as Synergy for the remainder of this paper).

The second group received autoclaved drinking water with no addition of probiotic. The prebiotic chosen for treatment is a combination long-chain inulin and short-chain oligofructose (FOS) in a mixture of 1:1. A fructo-oligosaccharide was chosen as it has been better characterized than other prebiotics. The combination of long and short chain was used so that prebiotic fermentation would occur more evenly along the length of the intestine. Fructo-oligosaccharides are broken down by bacterial enzymes before being transported into bacterial cells. Fructo-oligosaccharides with a degree of polymerization greater than 10 are fermented half as quickly as those with a DP less than ten[7]. It is hypothesized that the shorter FOS would be broken down earlier in the intestine; inulin would be more available for bacterial fermentation in the more distal segments of the colon relative to FOS. A dose of 7.5g/kg bodyweight per day was chosen based on a previous study[8]. It was added to drinking water for ease of administration; it could not be dissolved in a small enough volume to administer by pipette or oral gavage. Addition of the prebiotic into powdered food had previously been attempted, and was deemed to be a less exact method for measuring prebiotic intake. Autoclaved water was used to reduce bacterial growth in water bottles. The prebiotic-water was made fresh daily and given in the late afternoon and replaced in the early morning with regular autoclaved water, again, to prevent bacterial growth in the water bottles. The amount of prebiotic to be added to the drinking water was determined by weekly measurements of rat weights and daily measurement of water consumption. The rats received treatment for 4 weeks and were euthanized at 8

weeks of age, when inflammation and permeability alterations are reported to occur. Rat weights were compared using two-way ANOVA to determine differences between non-transgenic and HLA-B27 TG rats, and student's T test was used to determine differences between untreated and Synergy-treated rats in each group. Water and food consumption were compared using one-way ANOVA or student's T test where appropriate.

A second, low dose, study was carried out. A dosage of 0.3g/kg bodyweight per day of Synergy was administered. Prebiotic studies in humans report dose-dependent symptoms including abdominal pain, bloating and flatulence[9]. The low dosage used in this study was based on a common and somewhat tolerated dosage of 15g per day in humans. This was administered to the rats by pipette feeding. This is a more exact method of oral administration than addition into drinking water or food. Rats in the control group were pipette-fed water. Rats were treated for four weeks beginning at four weeks of age. Rat weights were compared using two-way ANOVA to determine differences between non-transgenic and HLA-B27 TG rats, and student's T test was used to determine differences between untreated and Synergy-treated rats in each group.

3.2.3. Organ Collection

At 8 weeks of age, rats were euthanized with carbon dioxide. Rats' abdomens were rinsed with ethanol and a transverse incision was made to expose the intestine and internal organs. The mesenteric lymph nodes were removed under sterile conditions and placed in phosphate-buffered saline (PBS) and kept on ice for isolation of lymphocytes. The cecum was excised and samples were taken for permeability assessment by Ussing chambers, histology, and cytokine analysis by enzyme-linked immunosorbant assay (ELISA). The colon was excised 1cm from the cecum to the rectum, and approximately 20cm of the terminal ileum were excised 1cm from the cecum. Samples were collected for permeability, histological assessment, and cytokine analysis (Figure 3.1).

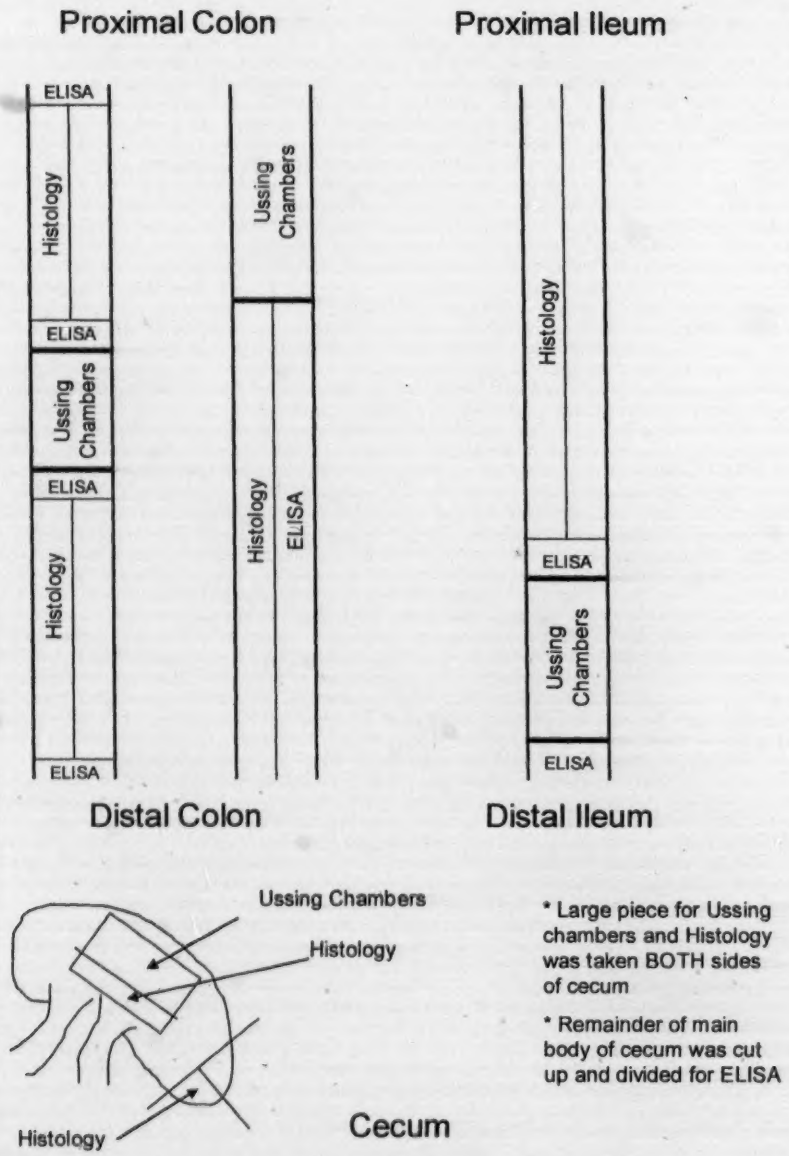

Figure 3.1. The segmentation of the intestinal tissues for histology, permeability, and cytokine measurements.

3.2.4. Histology

Tissues were fixed in a 10% acetate-buffered formalin solution for at least 24 hours. Organs were embedded in paraffin, sectioned at 6μm thickness, and stained with hematoxylin and eosin. Samples were evaluated by a single observer (L.A. Dieleman) using light microscopy. Inflammation was scored by a histological score as described previously[10] (Table 3.1). Briefly, the score was determined by the presence of inflammatory cells, a decrease in goblet cells, in increase in mucosa thickening and submucosa cell infiltration, and destruction of the architecture. The presence of ulcers and crypt abscesses was also taken into account. Statistical analysis was performed using the Mann-Whitney U Test, a non-parametric test based on the ranks of scores.

Table 3.1. Histological score to quantify the degree of gastrointestinal inflammation in HLA-B27 transgenic rats

	Score					Add
Criterion	0	1	2	3	4	+0.5–+1 for each
Inflammatory cells	–	↑	↑↑	↑↑↑	↑↑↑	ulcer
Goblet cells	–	↓	↓↓	↓↓↓	↓↓↓	crypt abscess
Mucosa thickening	–	↑	↑↑	↑↑↑	↑↑↑	
Submucosa cell infiltration	–	–	↑	↑↑	↑↑↑	
Destruction of architecture	–	–	–	↑	↑↑	

↑, increased; ↓, decreased.

From Rath, et al.[10]

3.2.5. Determination of IL-1β

Preparation of tissue homogenate

Ileal, cecal, and colonic tissue were homogenized in protease inhibitor cocktail (Sigma-Aldrich Canada Ltd., Oakville, ON, CAN) reconstituted in PBS and contained phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich Canada Ltd., Oakville, ON, CAN). Samples were then centrifuged at 4000rpm for 10 minutes. The supernatant was collected and centrifuged again for 5 minutes at 10 000rpm. Supernatant was again collected. Protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Mississauga, ON, CAN), and samples were diluted to 1-2 mg/mL protein.

IL-1β ELISA

IL-1β concentrations were determined with ELISA (R&D Systems, Inc. Minneapolis, MN, USA). Samples were run in triplicate. All steps in the ELISA were performed at room temperature. Wells were coated with 100µl goat anti-rat IL-1β antibody and incubated overnight. Plates were blocked in the morning for at least one hour. Diluted samples were added and incubated for two hours. Detection antibody (biotinylated goat anti-rat IL-1β) was incubated for two hours. Streptavadin-horseradish peroxidase (SAv-HRP) was added and incubated for 20 minutes. Substrate solution (3,3',5,5'-tetramethylbenzidine, TMB) was added (Bio-Rad Laboratories, Mississauga, ON, CAN) and allowed to develop. Stop solution (1M H_2S0_4) was added, and the absorbance determined at 450nm

(Wallac Instruments). IL-1β concentrations were standardized to the protein concentrations of each sample. Statistical analysis was performed using the Student's t-test.

3.2.6. Determination of IFN-γ

Preparation of fecal bacterial lysates

Prior to euthanization, a fecal bacterial lysate (FBL) solution was prepared using a method previously described for preparing cecal bacterial lysate[11, 12]. Briefly, stools from several transgenic (TG) rats were solubilized by vortexing in RPMI and incubated with 10μg/ml DNA-ase, 0.01M MgCl and then homogenized for 3 minutes using 0.1mm glass beads in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK). The lysate was centrifuged at 10 000g for 10 minutes, and the supernatant collected. This was repeated to remove glass beads and unlysed cells and was filtered through a 0.45μM syringe filter. Sterility was confirmed by aerobic and anaerobic culture. The lysate contains fecal contents free of live bacteria. It contains antigens present in the luminal contents that would be sampled by the intestinal immune system and presented to immune cells such as T lymphocytes. Sterility is necessary for culturing with immune cells for the time required to stimulate cytokine secretion.

Mesenteric lymph node cell cultures

At necropsy, mesenteric lymph nodes (MLN) were removed and single cell suspensions were prepared by gentle teasing[8]. MLN cells were washed twice and resuspended in RPMI supplemented with gentamicin and 5% fetal calf serum. 2×10^5 cells/well were cultured in a 96 well flat bottom microplate, in 0.2mL complete medium. Cells were stimulated with 10μg/mL and 50μg/mL FBL to stimulate cytokine secretion[12]. Cells were incubated at 37°C for three days, and supernatants were collected and stored at -70°C.

IFN-γ ELISA

Samples were diluted appropriately in RPMI and IFN-γ concentrations were determined by ELISA (BD Biosciences, San Diego, California, USA). Wells were coated with anti-rat IFN-γ monoclonal antibody and incubated overnight at 4°C. All incubations following this were performed at room temperature. The plate was blocked for at least one hour. Standards and samples were added and incubated for two hours. Biotinylated anti-rat monoclonal antibody was added and incubated for two hours. Streptavadin-horseradish peroxidase (SAv-HRP) was incubated for 20 minutes. Substrate solution (3,3',5,5'-tetramethylbenzidine, TMB) was added (Bio-Rad Laboratories, Mississauga, ON, CAN) and allowed to develop. Stop solution, 1M H_2S0_4 was added, and the absorbance determined at 450nm (Wallac Instruments). Statistical analysis was performed using the Student's t-test.

3.2.7. Permeability determined by Ussing Chambers

Tissue samples from the ileum, cecum and colon were excised at necropsy and placed in cold Krebs buffer (in mmol/l: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 25 $NaHCO_3$, pH 7.35) with 10mmol/L fructose. The serosal layer of the tissue was removed to remove the myenteric nerve plexus to eliminate its influence on electrophysiological measurements. The tissue was then mounted in Lucite chambers exposing mucosal and serosal surfaces to 10mL of oxygenated Krebs buffer. The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10mmol/L) was added to the serosal and mucosal sides. Permeabiltiy was assessed by mannitol flux across the tissue. Mannitol is a non-metabolized monosaccharide that crosses the gut via the transcellular route and measures whole gut permeability. To measure the basal mannitol flux, 10µCi H^3-labeled mannitol was added to the mucosal side. Baseline short-circuit current and tissue ion conductance were measured after a 20-min equilibration period. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by contimuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT, USA) every 5 minutes, except for 5-10 seconds when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's law. PD is expressed as millivolts (mV), Isc as

microamperes per square centimeter ($\mu A/cm^2$), and G as millisiemens/cm^2 (mS/cm^2). Forskolin, an adenylate cyclase-activating agent, was added to the serosal side (10^{-5} mmol/L) to induce increases in short-circuit current. Epithelial responsiveness was defined as the maximal increase in short-circuit current to occur within 5 minutes of exposure to the secretagogue. Chloride flux in response to forskolin is reported as the difference between the averaged values of the four consecutive five-minute fluxes prior to the addition of forskolin, and the five-minute flux post stimulation. Statistical analysis was performed using the Student's t-test and one way analysis of variance (ANOVA) where appropriate.

3.3. Results: High Dose Synergy *in vivo* Study

3.3.1. Body Weight

There were no differences in the weight gain of the Synergy-treated rats when compared to untreated rats, and this was true for both non-TG rats as well as HLA-B27 TG rats (Figure 3.3.1). Water and food intake did not differ (Figures 3.3.2 and 3.3.3 respectively).

3.3.2. Histology

There were significant differences in the cecum and the proximal colon of HLA-B27 TG rats versus untreated rats, but there were no differences in the mid or distal colon (Figure 3.3.5). There was no inflammation in the ileum. The inflammation was characterized by an increase in the number of inflammatory cells present, a decrease in the number of goblet cells, mucosal thickening and submucosal cell infiltration, and in some sections, the architecture of the tissue was lost (Figures 3.3.4). A possible explanation is that the cecum is the area of the intestine in the rat where the greatest fermentation occurs. Thus, the cecum is where oligofructose and inulin are fermented, where the greatest number of probiotic *Bifidobacteria spp.* would be found acting to prevent and reduce inflammation. The reduction in inflammation in the proximal colon could be due to the beneficial bacteria and bacterial products moving into the proximal colon

and exerting the effects, and it could also be due to the prebiotic reaching this segment of the intestine and subsequently being fermented by the *Bifidobacteria* present there. There were no differences in the mid and distal colon. This reflects that the beneficial effects of the prebiotics are not reaching these areas. It could also be that by these segments of the colon, stool has already formed into pellets. Beneficial components are no longer "in solution" and are less available to interact with the surrounding mucosa. Future studies may want to investigate the recovery of prebiotic in the different segments of the intestine as well as the differential distribution of probiotic bacteria in these segments.

3.3.3. Determination of IL-1β

Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine released from activated macrophages and T_h1 cells. The HLA-B27 TG rat model exhibits an immune response similar to Crohn's disease and is characterized by a shift to the Th1-mediated immune response. Thus we investigated IL-1β as a measurement of inflammation. There was a significant reduction in IL-1β concentrations in the cecum of TG rats in response to Synergy treatment versus untreated rats (Figure 3.3.6). This correlates with the observation of reduced histological inflammation in the cecum of TG rats. There was a reduction in the IL-1β levels in the ileum and colon of TG rats as well, although the reduction in those tissues was not significant. Again, this is likely due to the fact that the cecum is the segment of

the intestine where the majority of fermentation occurs, and so is the area containing the most beneficial bacteria and bacterial components. Interestingly, the non-transgenic rats also exhibited a modest reduction in IL-1β concentration. This lends itself to the idea that the intestinal tissue is under constant immune activation in order to prevent invasion by microorganisms and other potentially harmful antigens. The healthy immune activation state maintains a balance of the T_h1-mediated response with the T_h2, T_{reg}, and T_h3- mediated responses, thus preventing an over-reaction to commensal bacteria and food antigens. The probiotic bacteria stimulated by the prebiotic[8] in the non-transgenic rats is likely modulating the immune response away from the T_h1-mediated response and more towards the T_{reg}, and T_h3- mediated responses.

3.3.4. Mesenteric Lymph Node Cell Cytokine Response

Mesenteric lymph nodes (MLNs) collected in this study drain the cecum and proximal colon[8], and so provide an excellent basin from which to collect immune cells for study. MLN cells were removed from the tissue surrounding the cecum and the cells were isolated and then cultured in the presence of fecal bacterial lysate (FBL). FBL contains the bacterial and food antigens as well as RNA to which these immune cells would be exposed *in vivo* to which they would respond. The cytokines secreted by MLN cells in response to stimulation by FBL indirectly reflect the type of cells present in the MLNs. HLA-B27 TG rats exhibit at T_h1-type cytokine profile, and T_h1 cells secrete IFN-γ when activated.

Therefore, IFN-γ secretion from MLN cells in response to FBL stimulation was measured as an indication of immune activation. We found a reduced secretion of IFN-γ in response to FBL in the Synergy-treated TG versus untreated rats (Figure 3.3.7). This was not a significant difference however; there was some difficulty in obtaining consistent results. If what we are observing is an actual reduction in IFN-γ secretion from MLN cells as Hoentjen et al. observed in a similar study[8], it would reflect reduced activation of the T_h1-mediated response. This would correlate with studies that saw decreased pro-inflammatory cytokines in response to probiotic treatment in experimental colitis[1, 13, 14].

3.3.5. Intestinal Permeability

Epithelial barrier function was measured with Ussing chambers. A decrease in permeability was observed in the terminal ileum, cecum, and proximal colon, but none of these were statistically significant (Figure 3.3.8 and Figure 3.3.9). Removing the serosal layer in this procedure also poses a challenge and can introduce experimental error. A previous study observed an increase in permeability of the distal colon when the serosa was removed. Thus consistent removal becomes very important. There is also the possibility that mechanically removing the serosa may affect tissue integrity; tears in the tissue sometimes occurred during this step. There even seemed to be a difference in ease of serosal removal between inflamed tissues and non-inflamed tissues which may contribute to the experimental error. If we are observing an actual reduction in

mannitol flux, this could reflect a decrease in inflammation or a decrease in the tissue surface area. A decrease in surface area reflects fewer or blunted villi, and can be observed in disease states such as celiac disease[15]. If permeability is truly reduced in our Synergy-treated animals, it is more likely due to a reduction in inflammation rather than a reduction in surface area. Synergy-treated rat tissue demonstrated less inflammation histologically, and this was correlated with lower IL-1β levels. Histology samples showed increased tissue infiltration with immune cells (likely including neutrophils and T_h1 cells) in untreated rats versus Synergy-treated rats. Tissues infiltrated with neutrophils exhibit a down-regulation of tight junction (TJ) proteins[16]. A way to confirm neutrophil infiltration would be to test the tissue for myeloperoxidase, a characteristic enzyme of neutrophils. IFN-γ (secreted by T_h1 cells) has also been shown to increase permeability by disassembly of the TJ complex[17]. There also appears to be a trend towards a decrease in IFN-γ with prebiotic treatment. So taken together, a decrease in immune cell infiltration of the tissue and decreased IFN-γ correlate well with a decrease in permeability in response to Synergy treatment.

3.3.6. Electrophysiological parameters

Electrophysiological measurements were made using Ussing chambers, and short-circuit current (Isc), potential difference across the tissue (PD), conductance (G), and cAMP-mediated chloride secretion were determined. No

differences were found in Isc, PD, or G (Table 3.3.1). This is contrary to a previous study that compared HLA-B27 TG rats to non-TG controls using similar methods as in our study. This previous study found that Isc and conductance were reduced in HLA-B27 TG rats (PD was measured but results not given in study)[18].

There are several possibilities as to why the current study did not find any significant differences in these parameters where other studies have. Experimental error with respect to removal of the serosa is one possibility. Differences in animal colonies, especially in terms of the intestinal microflora, may also partially explain differences between our results and those in other facilities.

Short-circuit current

Ion flow, as reflected by the short-circuit current (Isc), is a measurement of the net movement of ions across the tissue per unit of time. It is a reflection of the absorptive and secretory capacity of the tissue[19]. In chronic inflammation, such as the HLA-B27 TG model of colitis, short-circuit current is reduced in comparison to non-TG controls[18].

Potential difference

Potential difference (PD) across the tissue represents the ability of the tissue to maintain the ion concentration gradients across the tissue, and indirectly represents the activity of the Na^+/K^+-ATPase pump. It is primarily determined by sodium transport. During inflammation, the epithelial barrier becomes leaky due to alterations of the tight junctions between the cells, and this will affect the transepithelial voltage; the more permeable the epithelial membrane, the lower the PD value. The PD is also affected by the Na^+/K^+-ATPase, which has reduced expression in the basolateral membrane of intestinal epithelial cells during inflammation[20], and this will also result in a lower PD value.

Conductance

Conductance is calculated using Ohm's law and is the reciprocal of the resistance of the tissue. It is a reflection of the passive ion movement across the tissue. Paracellular ion movement is the major contributor to this measurement and is dependent on the number and integrity of tight junctions between the cells, although transcellular ion movement also contributes.

cAMP-mediated chloride secretion

Chloride secretion through the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) plays an important role in colonic electrolyte balance and appears to be a critical component for determining transepithelial ion transport[21]. The CFTR is activated by phosphorylation by protein kinase A (PKA) which is activated in response to an increase in intracellular cyclic adenosine monophosphate (cAMP) levels, which is stimulated by hormone an neurotransmitter signaling[21]. Forskolin activates adenylate cyclase, an enzyme which dephosphorylates adenosine triphosphate (ATP) to form cAMP. Thus, forskolin is able to stimulate CFTR-mediated chloride secretion. Under chronic inflammatory conditions in the intestine, chloride secretion in response to forskolin is reduced.

cAMP-mediated chloride secretion is the only electrophysiological parameter in this study that had any significant differences. There was a significant reduction in forskolin-stimulated Cl^- secretion in the HLA-B27 TG rats versus non-TG rats in the cecal tissues (Table 3.3.1). There also appeared to be a trend towards an increase in chloride secretion in the Synergy-treated rats versus untreated rats, indicating a possible reduction in inflammation and restoration of process governing the regulation of the CFTR. Future studies in this model may want to investigate intracellular levels of cAMP as well as CFTR expression, especially that of the apical membrane.

3.3.7. Experimental Results: High Dose Synergy

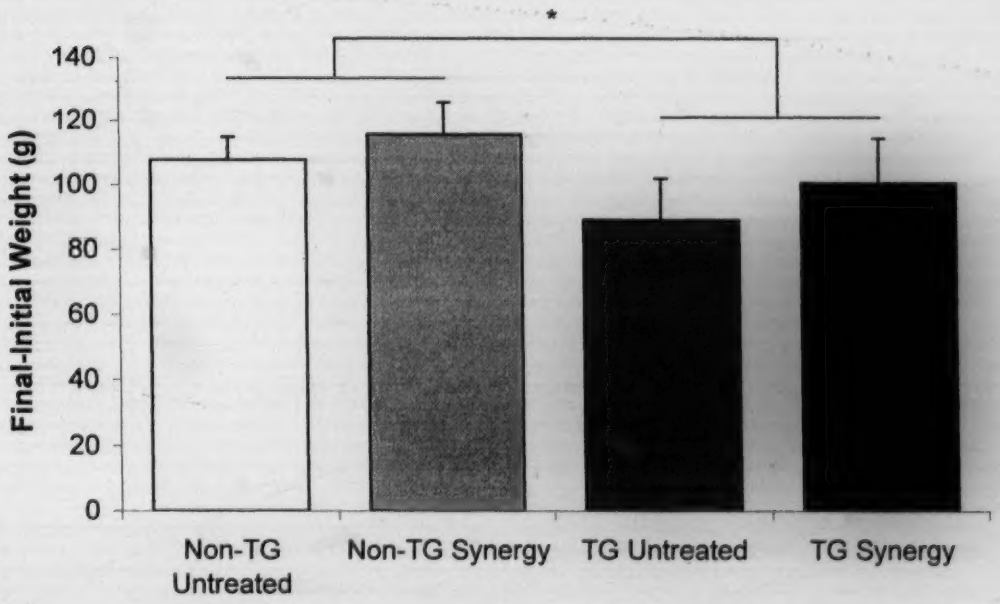

Figure 3.3.1. Synergy treatment effects on weight gain.

Non-transgenic (non-TG) rats gained more weight over the course of the study than the HLA-B27 TG rats ($p=0.04$). There was no difference in weight gain due to treatment in the non-TG group ($p=0.12$) or in the HLA-B27 TG group ($p=0.2$). Non-TG Untreated ($n=4$), Non-TG Synergy ($n=5$), TG Untreated ($n=6$), TG Synergy ($n=8$). Data are presented as the average ± standard deviation.

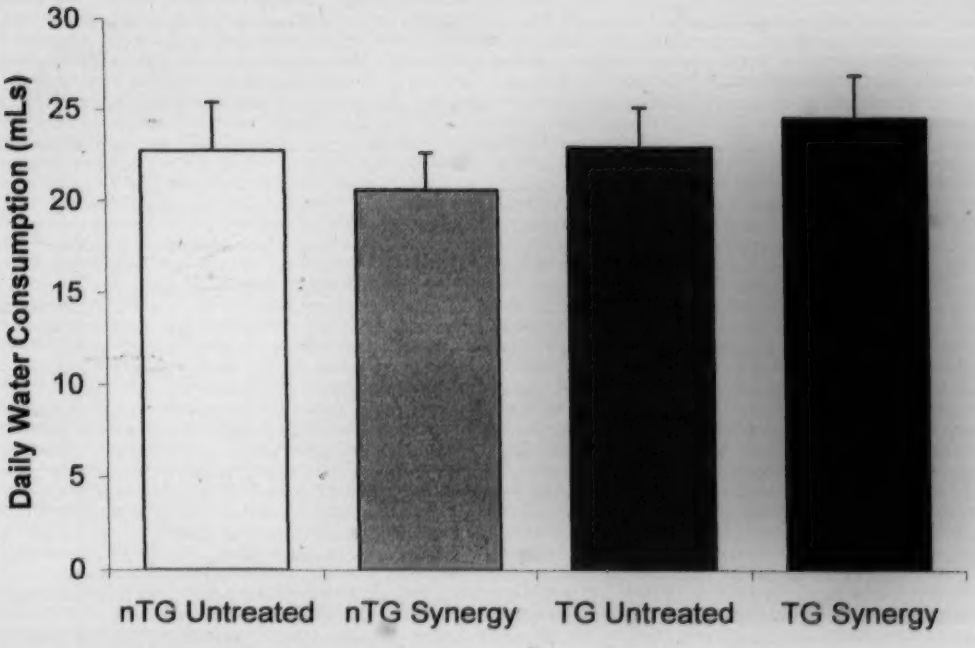

Figure 3.3.2. Daily water consumption of rats.

Daily water consumption of the rats did not differ among the groups ($p=0.12$). Synergy treatment did not affect water consumption in the non-TG group ($p=0.3$) or in the TG group ($p=0.3$). Daily water consumption was calculated using 3 cages per group over the entire course of treatment (4 weeks). Data are presented as the average ± standard deviation.

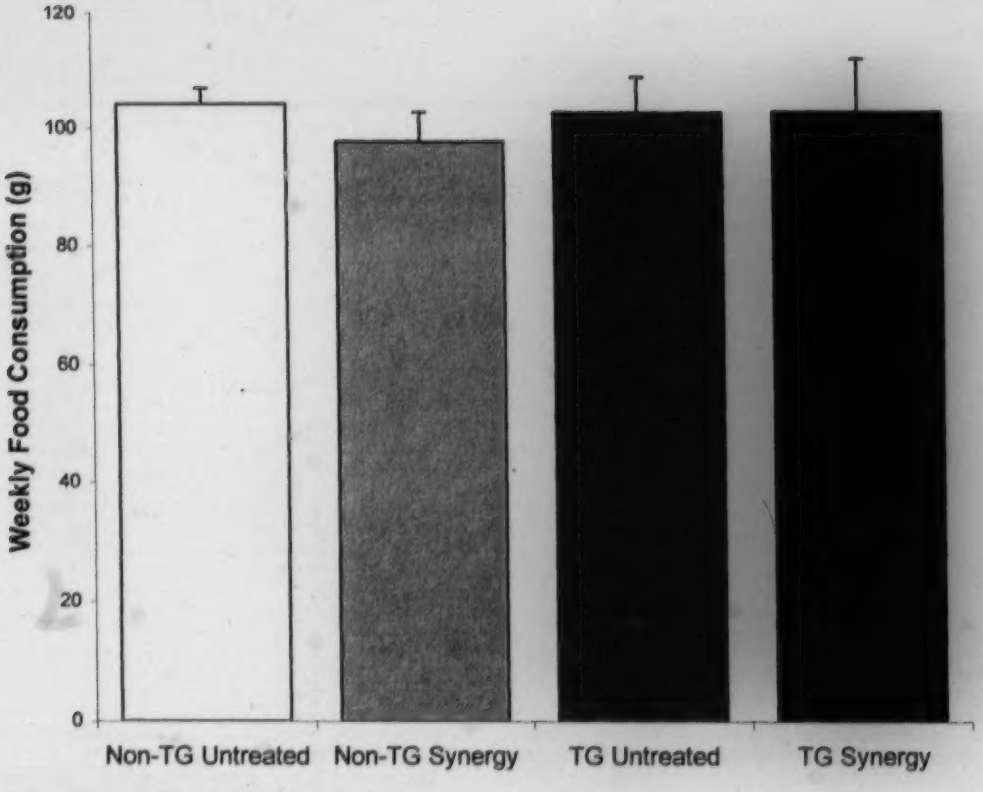

Figure 3.3.3. Synergy treatment effects on food consumption.

Synergy treatment did not affect weekly food consumption (p=0.6). There was no difference due to treatment in either the non-TG rats (p=0.1) or the TG rats (p=0.96). Non-TG Untreated (n=3), Non-TG Synergy (n=5), TG Untreated (n=3), TG Synergy (n=3). Data are presented as means ± standard deviation.

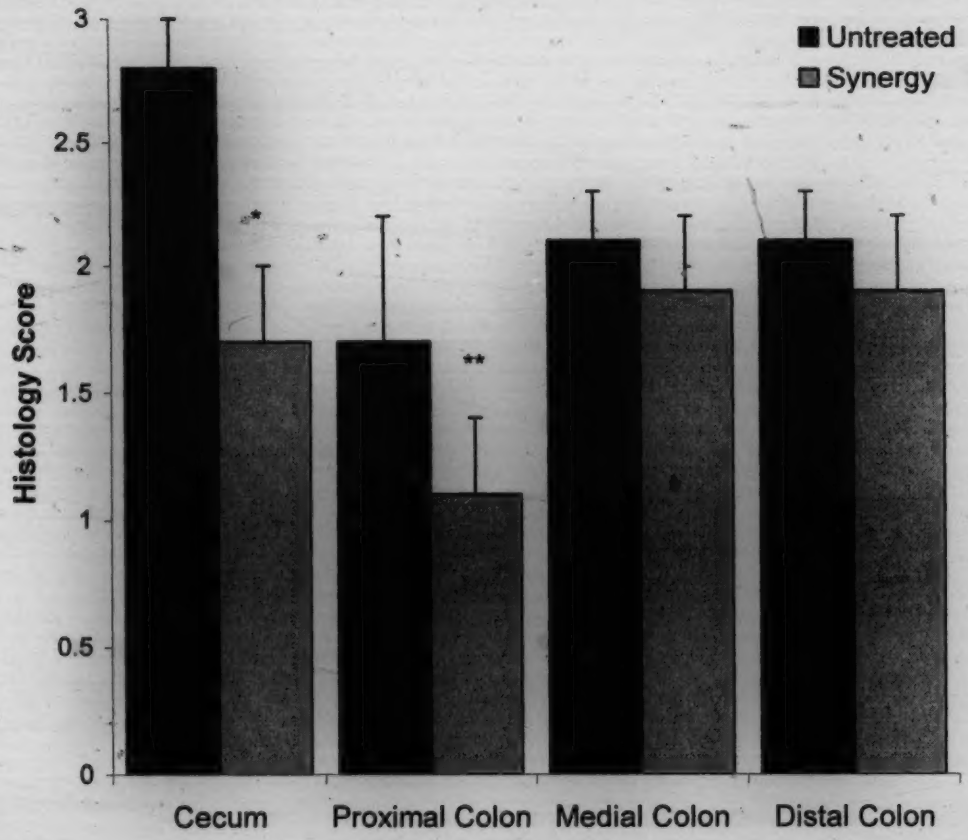

Figure 3.3.4. Histological scores of transgenic rat intestinal tissues.

The histological scores in the cecum and proximal colon were significantly lower in the Synergy-treated group versus the untreated rats (*p=0.001 versus untreated TG rats, **p=0.002 versus untreated rats). There was a trend towards a lower score in the mid colon and distal colon, however this decrease was not significant. Untreated (n=8), Synergy (n=7). Values are presented as mean ± standard deviation.

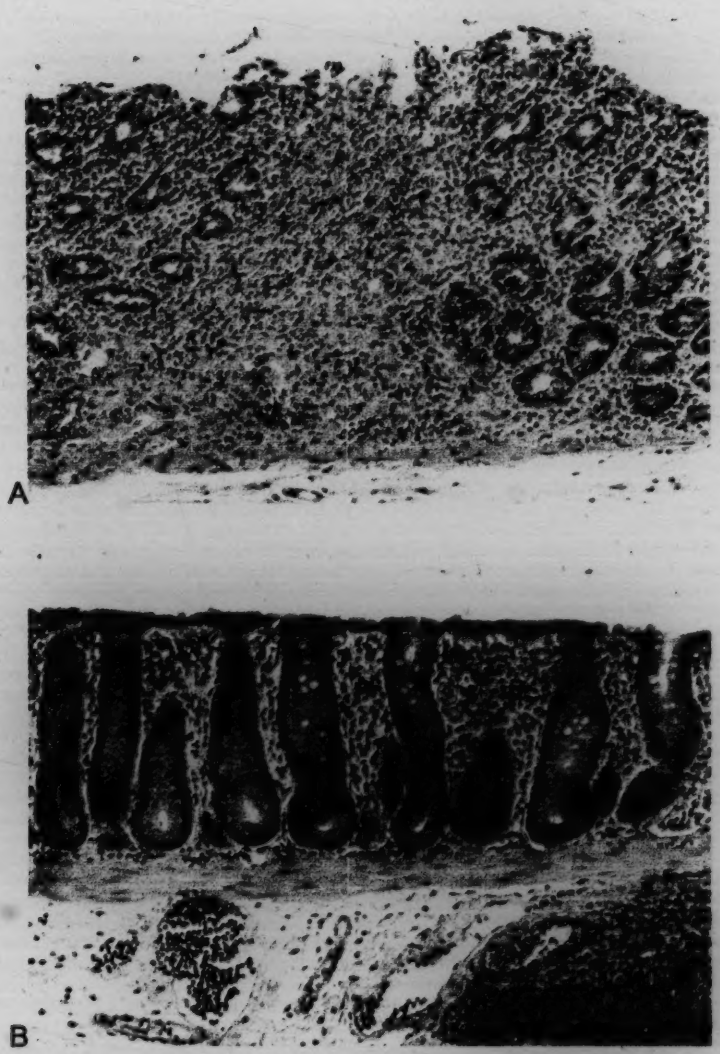

Figure 3.3.5. Histology photomicrographs of the ceca of HLA-B27 TG rats.

Representative photomicrographs of cecal tissue sections (40x) from the ceca of 8-week-old HLA-B27 TG rats treated for 4 weeks with water (A) or prebiotic Synergy (B) prior to the development of inflammation.

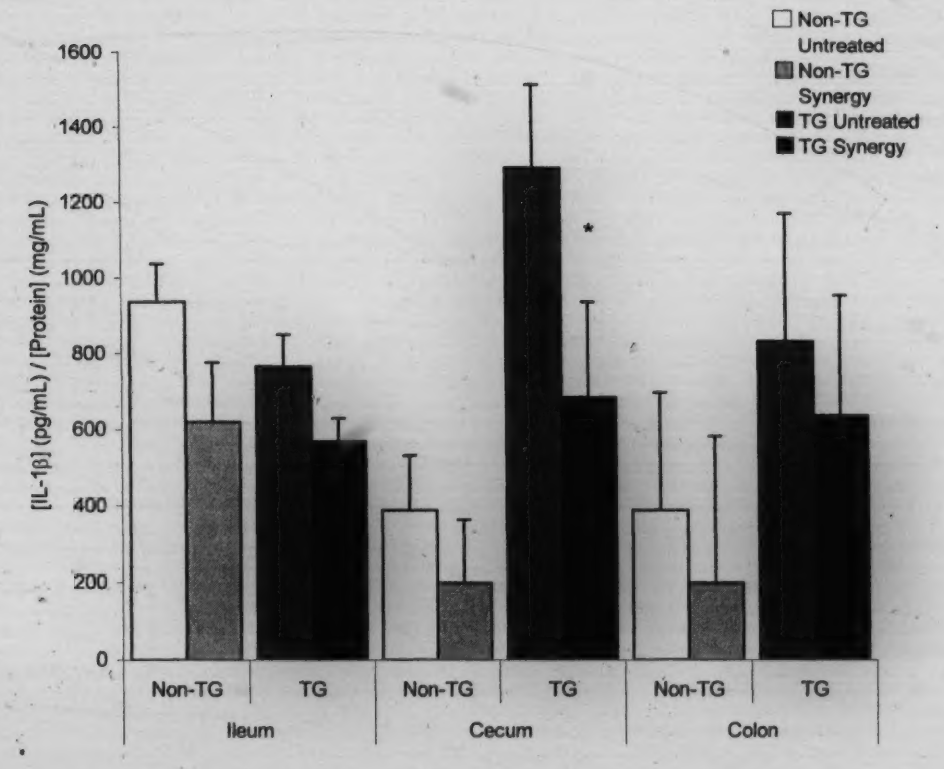

Figure 3.3.6. Interleukin-1β (IL-1β) concentrations in intestinal tissue homogenates of non-transgenic and HLA-B27 TG rats.

IL-1β was significantly reduced in the cecal tissue of HLA-B27 TG rats treated with Synergy versus their untreated counterparts (*$p<0.001$). There was a trend towards reduction of IL-1β in the other tissues, but these values did not reach significance. IL-1β concentrations were measured in triplicate by ELISA and standardized using protein concentration. Ileum: nTG unt (n=3), nTG Syn (n=5), TG unT (n=5), TG Syn (n=8); Cecum: nTG unt (n=6), nTG Syn (n=9), TG unT (n=9), TG Syn (n=11); Colon: nTG unt (n=8), nTG Syn (n=7), TG unT (n=10), TG Syn (n=13). Values are expressed as the mean ±SEM.

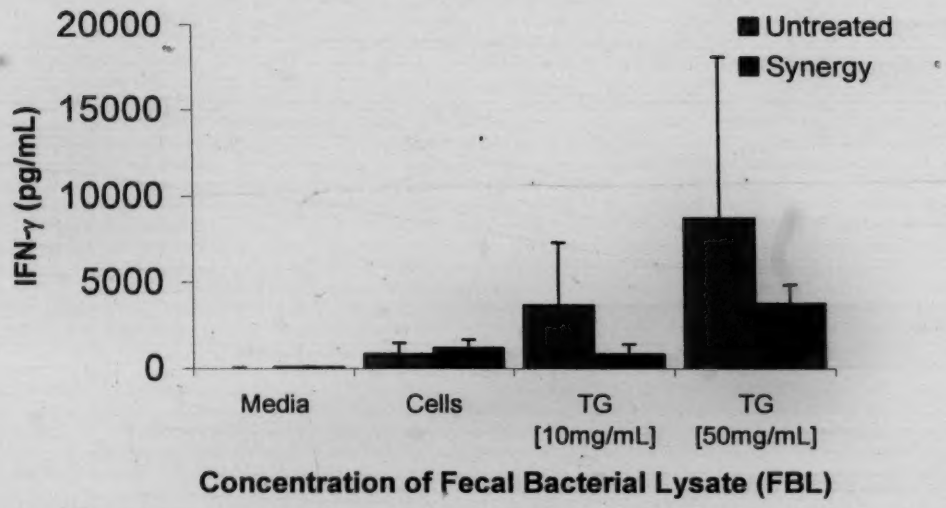

Figure 3.3.7. Interferon-γ (IFN-γ) secretion from MLN cells stimulated with TG fecal bacterial lysate (FBL).

There appears to be a trend towards a reduction in the IFN-γ secreted by Synergy-treated rat MLN cells versus untreated rat MLN cells, however there was no significant difference at any of the concentrations of FBL tested (10 mg/mL and 50 mg/mL protein concentration, collected from TG rats). Media, no cells (p=0.4), Cells only (p=0.5), 10 mg/mL (p=0.3), 50 mg/mL (p=0.4). Each group consisted of three rats. Values are presented as mean ± standard deviation.

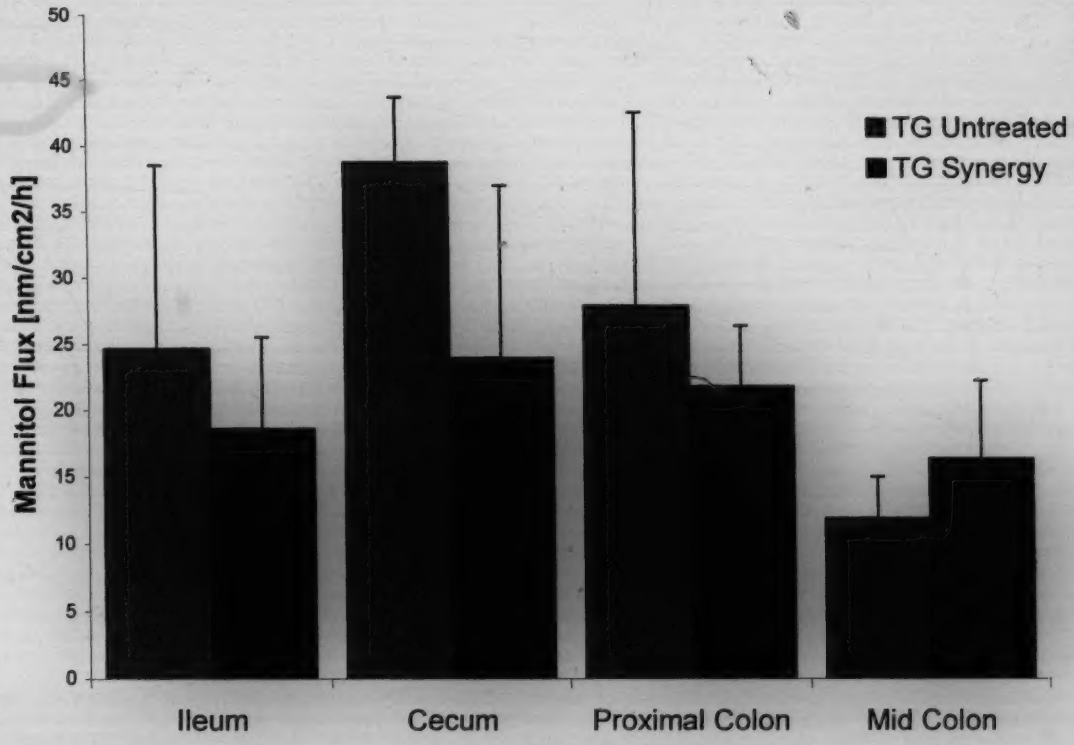

Figure 3.3.8. Intestinal permeability of HLA-B27 TG rat intestinal tissues.

There is a trend towards reduced permeability in the ileum, cecum, and proximal colon of rats treated with Synergy as compared to their untreated counterparts, although no significant differences were found. Ileum (p=0.652), cecum (p=0.5), proximal colon (p=0.45), mid colon (p=0.3). Ileum: unT (n=2), Syn (n=2); cecum: unT (n=4), Syn (n=4); proximal colon: unT (n=5), Syn (n=4); mid colon: unT (n=3), Syn (n=5). Values are presented as mean ± standard deviation.

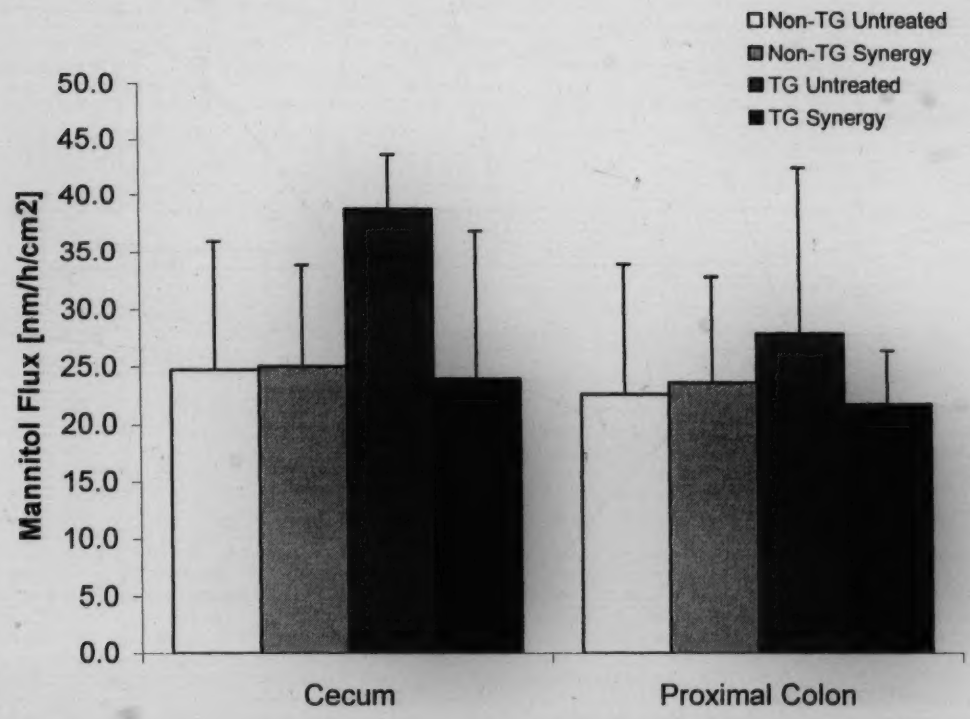

Figure 3.3.9. Epithelial barrier integrity in tissue from the cecum and proximal colon of HLA-B27 TG rats.

There appears to be a trend towards a reduction in permeability in the cecum in prebiotic-treated TG rats versus untreated rats. This difference was not statistically significant however (p=0.3). There was no significant difference observed in the proximal colon (p=0.8). Cecum: nTG unT (n=5), nTG Syn (n=2), TG unT (n=3), TG Syn (n=4); proximal colon: nTG unT (n=5), nTG Syn (n=3), TG unT (n=5), TG Syn (n=4). Values represent the average ± standard deviation.

Table 3.3.1. Electrophysiological parameters.

There were no significant differences in electrophysiological parameters: short-circuit current (Isc), potential difference (PD), and conductance (G). There was a difference found in cAMP-mediated chloride secretion (Cl^-) in the cecum of non-TG versus TG rats, however there were no differences between untreated and Synergy-treated rats (non-TG: p=0.6, TG: p=0.7).

Tisue	Type	Average (Std Dev)			
		Isc	PD	Conductance	Cl^-
Ileum	TG UnT	17.6 (4.3)	0.7 (0.3)	29.2 (14.3)	23.0 (10.7)
	TG Syn	17.2 (7.0)	0.7 (0.2)	24.5 (5.3)	39.3 (18.7)
Cecum	nTG UnT	57.5 (14.5)	2.3 (0.8)	26.6 (3.5)	102.8 (34.6)
	nTG Syn	65.7 (37.9)	3.2 (1.8)	29.3 (7.2)	91.3 (17.0)
	TG UnT	60.8 (17.1)	2.1 (0.3)	26.6 (8.3)	30.2 (6.9)
	TG Syn	38.6 (25.0)	2.0 (0.6)	32.3 (12.3)	40.2 (48.4)
Proximal Colon	nTG UnT	50.8 (11.2)	1.4 (0.8)	30.4 (12.3)	103.8 (55.1)
	nTG Syn	34.1 (7.7)	1.6 (0.8)	23.9 (6.6)	23.2 (18.5)
	TG UnT	39.0 (17.2)	1.3 (1.0)	30.2 (12.2)	36.4 (38.4)
	TG Syn	28.1 (20.2)	0.9 (0.4)	29.9 (9.2)	64.9 (52.2)
Mid Colon	TG UnT	8.8 (4.7)	0.6 (0.3)	15.3 (1.6)	10.7 (5.5)
	TG Syn	8.8 (5.5)	0.4 (0.1)	22.5 (18.0)	16.5 (13.6)

3.4. Results: Low Dose Synergy *in vivo*

3.4.1. Body Weight

There were no differences in weight gain in the rats treated with a low-dose of Synergy (Figure 3.4.1).

3.4.2 Histology

There was no significant difference observed in the histological scores of untreated versus Synergy-treated HLA-B27 TG rats. It appears as though there may have been slight improvement (Figure 3.4.2), and this may have become significant with a longer treatment time-course or possibly with a greater number of rats in the treatment groups. There was no histological inflammation in the ileal tissues, which is consistent with our findings in the high-dose Synergy experiment as well as in previous studies.

3.4.3. Determination of IL-1β

There were no significant differences observed between low-dose Synergy-treated HLA-B27 TG rats and their untreated counterparts (Figure 3.4.3). This correlates with the histological findings.

3.4.4. Permeability and Electrophysiological Parameters

Low-dose Synergy had no effect on the permeability of the ileum and mid colon of non-TG and HLA-B27 TG rats (Figure 3.4.4). There were also no differences in any of the electrophysiological measurements including conductance, short-circuit current, chloride secretion in response to forskolin, or transepithelial voltage (Table 3.4.1). This is similar to the findings in the high dose experiment, where we also did not observe any differences in the ileum and mid colon. In the high-dose group, we attributed this absence of a difference to the location of the tissues collected; measuring the cecum and proximal colon may have shown a possible effect of low dose Synergy. However in light of the fact that there were also no differences in the histological scores or in IL-1β, it is more likely that the low-dose of Synergy is not a high enough dose to affect the inflammation in the intestinal tract of HLA-B27 TG rats.

3.4.5. Low Dose Results

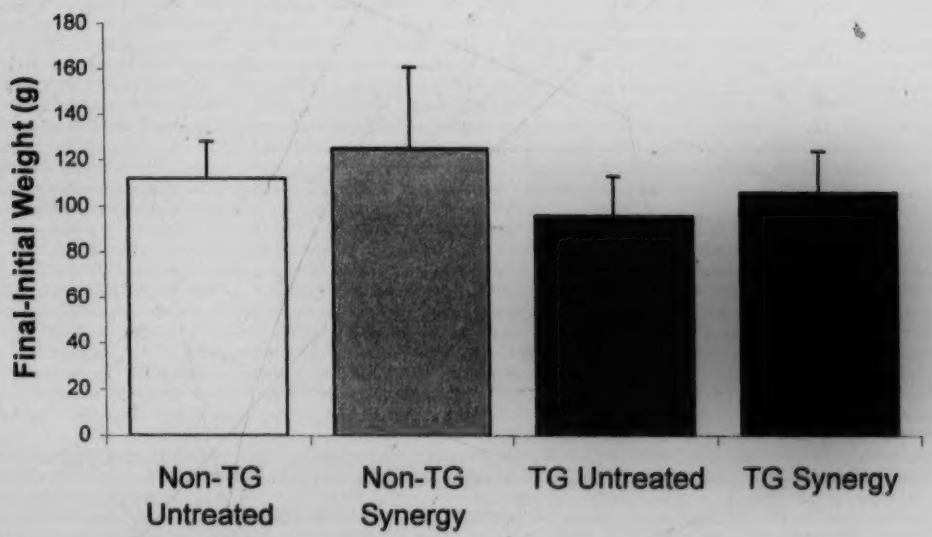

Figure 3.4.1. Low dose Synergy treatment effects on weight gain.

Initial body weights were similar among all groups (p=0.3), and there was no difference in weight gain between non-transgenic rats and HLA-B27 TG rats (p=0.15). Prebiotic treatment with Synergy did not significantly increase body weight in either the non-TG (p=0.3) or TG (p=0.3) groups. Non-TG Untreated (n=7), Non-TG Synergy (n=6), TG Untreated (n=4), TG Synergy (n=4). Data are presented as means ± standard deviation.

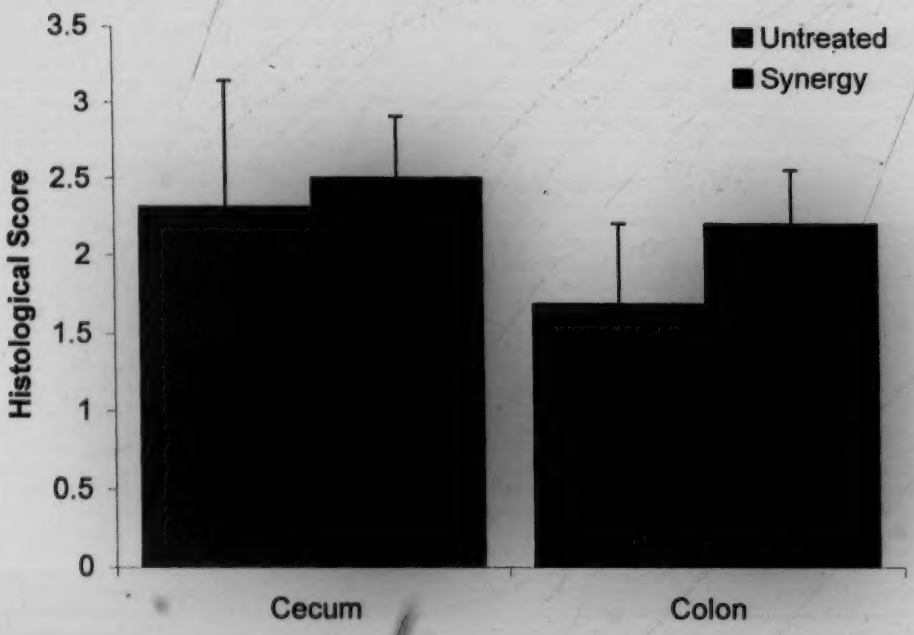

Figure 3.4.2. Histological scores of HLA-B27 transgenic rat intestinal tissues.

There were no significant differences in the histological assessment of inflammation in the ceca ($p=0.08$) and colons ($p=0.09$) between Synergy-treated rats and their untreated counterparts. There was no evidence of histological inflammation in the ileal tissues of either group (data not shown). There were 4 rats in each group. Data are presented as the mean ± standard deviation.

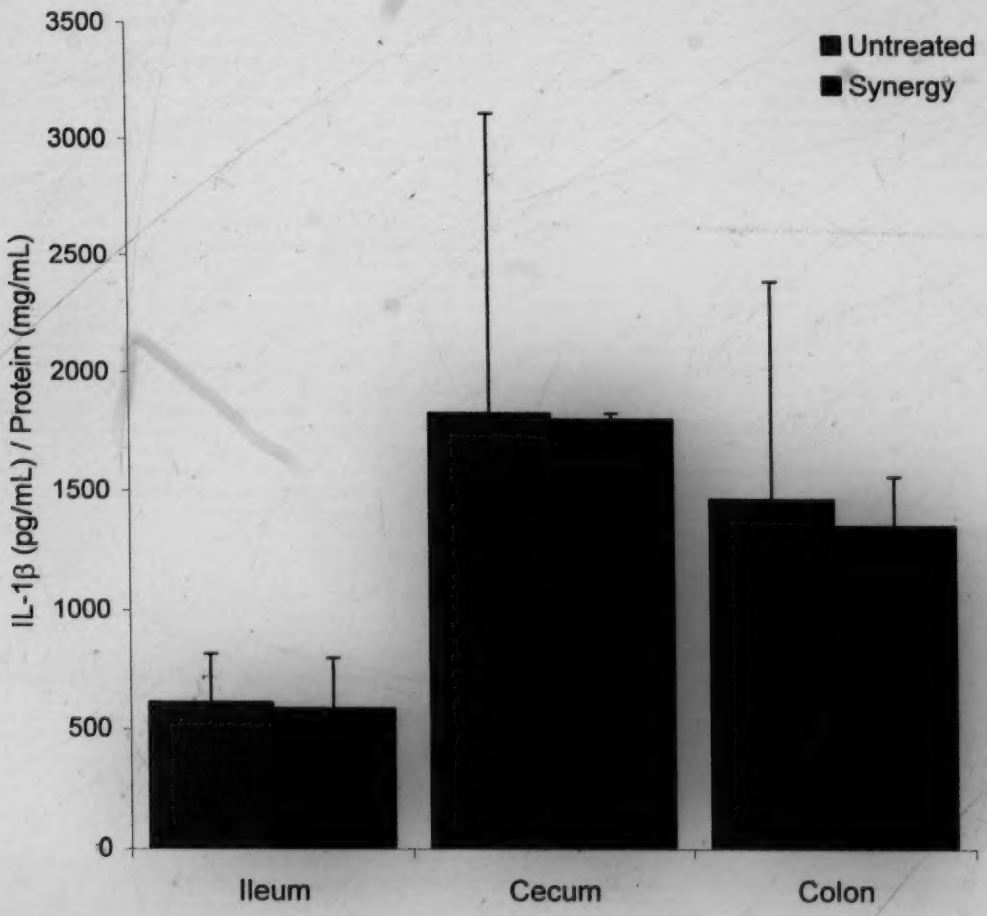

Figure 3.4.3. Interleukin-1β (IL-1β) concentrations in intestinal tissue homogenates of HLA-B27 TG rats treated with low dose Synergy.

There were no differences between the Synergy-treated rats and untreated rats. Ileum (p=0.9), cecum (p=0.8), colon (p=0.9). There were 3 or 4 rats in each group. Data are presented as the mean ± standard deviation.

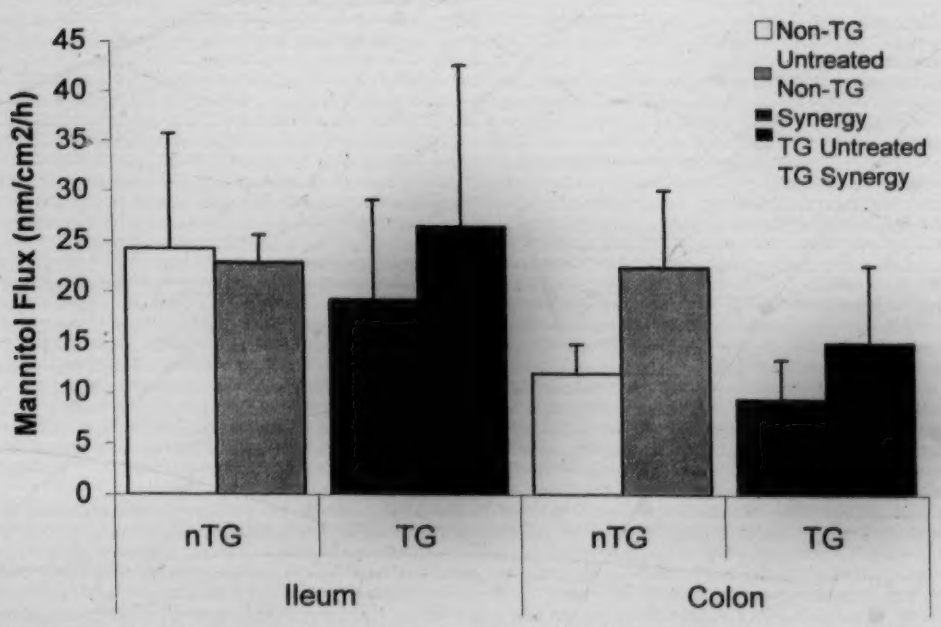

Figure 3.4.4. Permeability of rat intestinal tissues as measured with Ussing chambers.

There were no differences between Synergy-treated rats and their untreated counterparts in any of the groups. Ileum: non-TG (p=0.5), TG (p=0.2); Colon: non-TG (p=.12), TG (p=0.3). Ileum: nTG unT (n=4), nTG Syn (n=3), TG unT (n=2), TG Syn (n=3); Mid colon: nTG unT (n=5), nTG Syn (n=3), TG unT (n=4), TG Syn (n=3). Data are presented as the mean ± standard deviation.

Table 3.4.1. Electrophysiological parameters.

There were no significant differences in electrophysiological parameters: short-circuit current (Isc), potential difference (PD), conductance (G), and cAMP-mediated chloride secretion (Cl^-).

Tisue	Type	Average (Std Dev)			
		Isc	PD	Conductance	Cl^-
Ileum	nTG UnT	29.8 (14.7)	1.7 (0.1)	27.8 (13.3)	35.4 (21.4)
	NTG Syn	44.7 (14.2)	1.4 (0.3)	20.5 (12.0)	39.1 (7.8)
	TG UnT	31.0 (11.8)	1.4 (0.2)	9.3 (4.0)	59.4 (11.4)
	TG Syn	40.0 (9.7)	1.6 (1.0)	20.2 (9.7)	54.2 (33.0)
Mid Colon	nTG UnT	46.6 (39.1)	2.1 (1.6)	26.8 (10.0)	35.3 (27.5)
	NTG Syn	32.5 (20.2)	2.0 (0.6)	33.5 (11.5)	27.8 (12.9)
	TG UnT	9.3 (4.1)	0.7 (0.2)	26.6 (6.8)	9.7 (9.2)
	TG Syn	20.2 (4.8)	1.2 (0.6)	33.1 (15.1)	24.9 (13.5)

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Chapter 4: *In vitro* studies

4.1. Overview

Intestinal epithelial cells are in continuous contact with the intestinal microflora and luminal contents. They participate in mucosal defense by preventing entry of pathogens, secreting protective factors, acting as antigen-presenting cells, and expressing cytokines and chemokines in response to cytokines such as IL-1, TNF-α, and IFN-γ as well as in response to luminal antigens[1].

HT-29 cells were chosen for this study for their ability to secrete IL-8, a chemokine that attracts neutrophils, in response to pro-inflammatory signals such as TNF-α. Previous studies have shown that HT-29 cells incubated with probiotic strains secrete decreased amounts of IL-8 in response to non-pathogenic *E. coli* and pathogenic strains such as *Salmonella enterica* Dublin and *S. typhimurium*, as well as to bacterial components such as flagellin[2, 3]. Our *in vivo* study showed that there were beneficial effects to feeding the prebiotic combination Synergy to SPF HLA-B27 transgenic rats. Hoentjen et al. also showed that the prebiotic combination can reduce inflammation possibly by increasing probiotic bacteria such as *Bifidobacteria spp*. The aim of this study was to determine if the prebiotics themselves had anti-inflammatory properties independent of their ability to increase probiotic bacteria.

4.2 Methods

4.2.1 Cell Culture

HT-29 cells were obtained from American Type Culture Collection (Rockwell, MA) and cultured in RPMI 1640 (Gibco, Burlington, ON, CAN) supplemented with 10% heat-inactivated fetal calf serum (Cansera, Rexdale, ON, CAN), 2 mM glutamine1 mM sodium pyruvate, 2% sodium bicarbonate, 1x penicillin-streptomycin (Gibco) and 10mM Hepes. Confluent monolayers (passage 12-24) were used in all experiments.

Three prebiotic preparations, Raftilose, Raftiline, or Raftilose Synergy (all from Orafti, Tienen, Belgium), were added to RPMI with 10% FBS, warmed to dissolve pH-balanced to 7.4, and filtered through a 0.22μm syringe filter. The prebiotic solutions were diluted to 0.1%, 1.0% and 2.0% w/v. 200μL of the solutions were added to each well of a 96-well plate and incubated for 24 hours. Other studies have incubated probiotics on HT-29 cells between 1 and 32 hours[1, 4], so a 24 hour incubation period was chosen. After 24 hours, the prebiotic solutions were replaced with the same concentrations of Raftilose, Raftiline and Synergy dissolved in serum-free media, with or without 10ng/mL TNF-α (R&D Systems, Minneapolis, MN, USA) and incubated for 6 hours. Serum-free media was used to minimize experimental variability, and TNF-α concentration and duration were based on a previous study[5]. After incubation, the supernatant was collected, and the cells were washed twice with PBS.

4.2.2 Cell Viability Assay

Cell viability was measured to determine if IL-8 secretion changes were due to similar changes in the number of viable cells. It was measured with a colourimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT was diluted (1mL of 5mg/mL MTT in 5mL PBS) and 60μL were added to each well. The cells were incubated with MTT for 4 hours. 100mL of dimethyl sulfoxide (DMSO) was added to each well and incubated for 30 minutes to dissolve and release the formazan crystals. The absorbance was read at 570nm (and background subtracted at 650nm) using a Wallac plate reader.

4.2.3 Determination of IL-8 Protein

Supernatants from cell culture were measured via ELISA as follows: 96-well ELISA plates (Costar #3369) were coated with 0.25mg/mL IL-8 capture antibody (R&D Systems) in PBS (pH 7.4) overnight at 4°C. Plates were then blocked for 2 hours with blocking buffer (10% v/v newborn calf serum in PBS). Plates were washed three times between all steps with wash buffer (0.05% Tween 20 in PBS). Samples (100μL) and standards (0-4000pg/mL human recombinant IL-8; R&D Systems) were incubated on the plates for 2 hours. Biotinylated anti-IL-8 antibody (R&D Systems) was added (20ng/mL in PBS) and plates were incubated for 1 hour. Plates were incubated for 30 minutes with peroxidase-

streptavadin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in blocking buffer with 0.05% Tween 20 to a concentration of 62.5ng/mL. The plates were developed with 3,3',5,5'-tetramethylbenzidine (BioRad), stopped with acid (1M H_2SO_4), and the absorbance was immediately read at 450nm with an ELISA plate reader. All steps were carried out at room temperature.

4.3. Results: Prebiotic treatment of HT-29 cells

HT-29 cells are an intestinal epithelial cell line that is known for consistent secretion of the pro-inflammatory chemokine, IL-8, in response to stimuli such as TNF-α. Other studies have shown that probiotic strains were able to modulate immune responses in these cells and reduce the amount of IL-8 secreted in response to numerous pro-inflammatory stimuli[3]. Our high-dose Synergy study showed significant differences and many trends towards an improvement in inflammation, so we set out to determine if the prebiotics on their own, without probiotic bacteria, could reduce inflammation. HT-29 cells were treated for 24 hours with Synergy and the long and short chain components of it: Raftiline and Raftilose, respectively. Fructo-oligosaccharides are the best characterized type of prebiotics and were chosen to relate with the *in vivo* study. TNF-α is known to stimulate IL-8 secretion from HT-29 cells. It was added as a pro-inflammatory stimulus, and we measured the resulting IL-8 secretion as well as cell viability. Cell viability was determined to ensure that any changes in IL-8 secretion were not due to alterations in cell viability. There were no differences in cell viability (Figure 4.4.1) or IL-8 secretion (Figure 4.4.2) in response to any of the prebiotic

treatments at any of the concentrations tested, with or without TNF-α added. (Higher concentrations of prebiotic did not stay in solution and so were not tested.) We can conclude that there are no immunomodulatory effects on HT-29 cells due to short-chain oligofructose (Raftilose), long-chain inulin (Raftiline), or a combination of these (Synergy) on their own.

4.4. Results

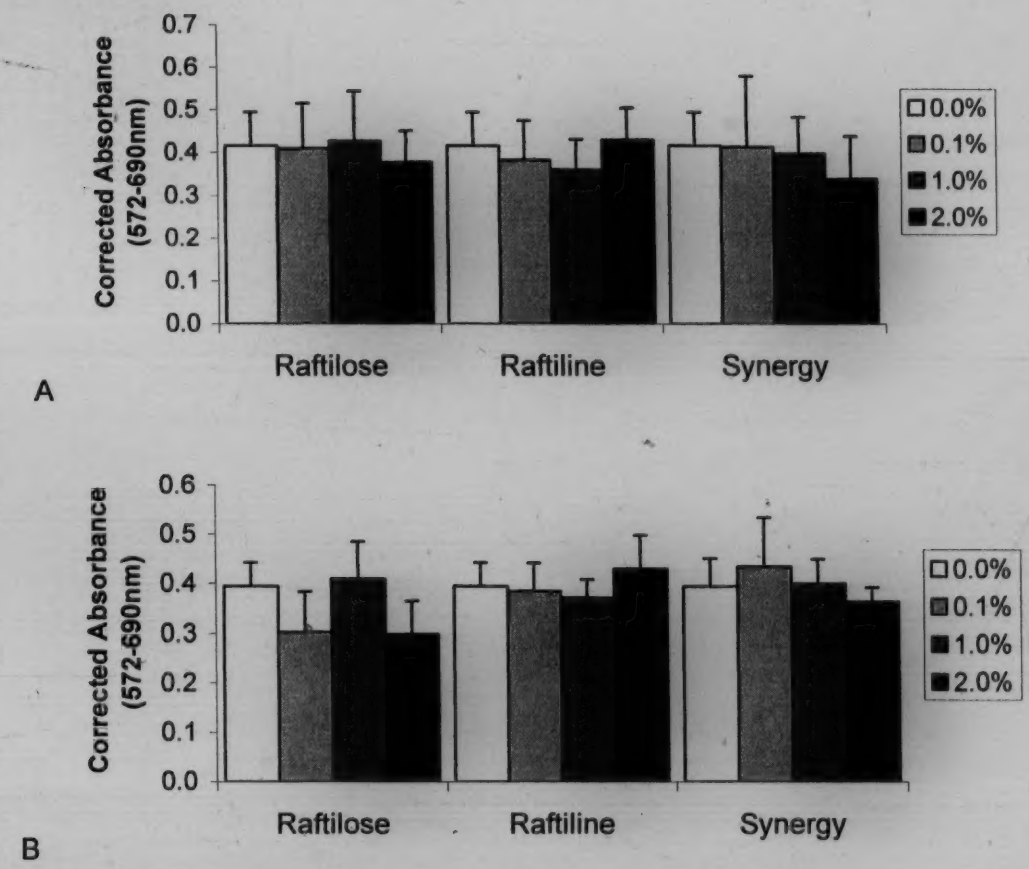
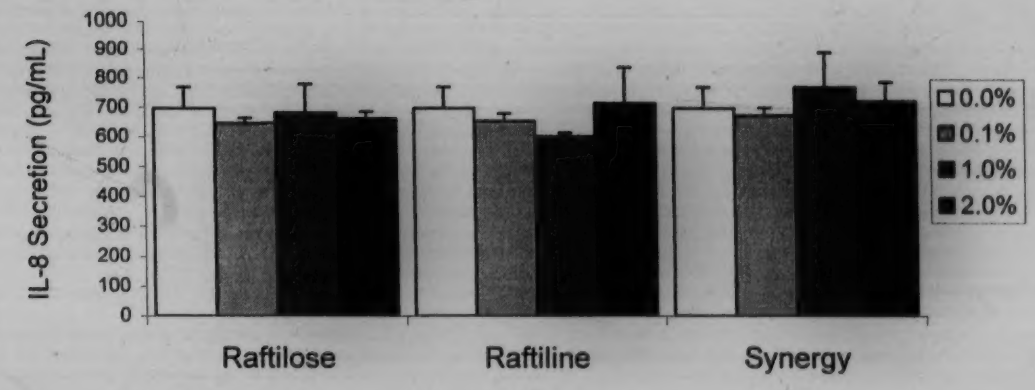


Figure 4.4.1 Cell viability as measured by MTT assay.

There were no differences in cell viability between the different concentrations of prebiotic with no TNF-α added (A) and with TNF-α added (B). No TNF-α added: Raftilose (p=0.8), Raftiline (p=0.3), Synergy (p=0.3). TNF-α added: Raftilose (p=0.11), Raftiline (p=0.3), Synergy (p=0.3). There were 8 samples in the untreated groups and 6 samples in the groups treated with TNF-α. The experiment was done twice. Samples are presented as the mean ± standard deviation.



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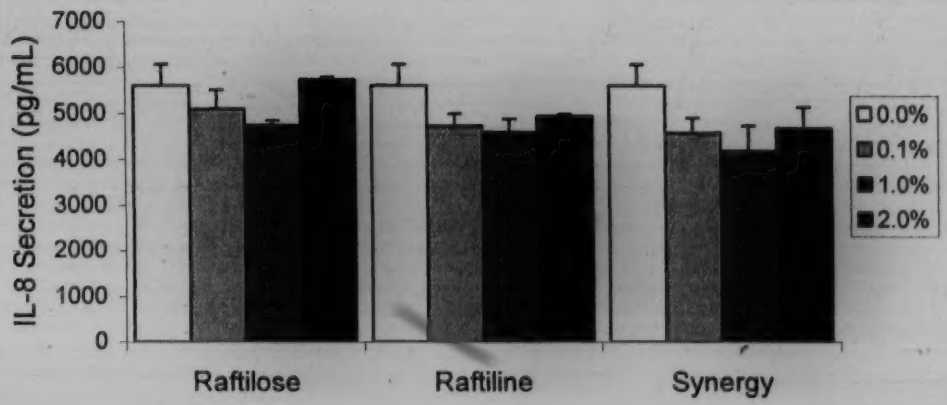

B

Figure 4.4.2. IL-8 secretion from HT-29 cells treated with prebiotic.

There were no differences in the IL-8 secretion in response to the different concentration of prebiotic used when (A) no TNF-α was added (Raftilose: p=0.8, Raftiline: p=0.3, Synergy: 0.5) or when (B) TNF-α was added (Raftilose: p=0.25, Raftiline: p=0.11, Synergy: p=0.10). There were three wells of HT-29 cells in each group and the experiment was done twice. Data are presented as the mean ± standard deviation.

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Chapter 5: Discussion

5.1 Overview of the project

The intestine contains a complex and diverse society of bacteria, and it has the task of determining if bacteria and antigens pose a potential risk, while still allowing for the uptake of nutrients. These pathogenic, non-pathogenic and probiotic organisms can modulate host health, and diet appears to be a major contributing factor regulating the concentration of species inhabiting the intestinal tract[1]. Prebiotics are non-digestible oligosaccharides that can be found in foods such as chicory root and in dietary supplements. Prebiotics promote the growth of probiotic strains of bacteria such as *Bifidobacteria* and *Lactobacilli*. Probiotics have been shown to prevent and reduce inflammation in IBD. This study evaluated the ability of the prebiotic combination of oligofructose and inulin, Synergy, to prevent inflammation in the HLA-B27 transgenic rat model of colitis. The second part of the study determined if prebiotics had any anti-inflammatory properties independent of probiotic bacteria. The high dose of Synergy prevented inflammation, and a trend towards improvement in permeability was observed. There were no differences in the low dose of Synergy versus placebo. The cell culture work determined that there are no effects on pro-inflammatory chemokine secretion or cell viability due to prebiotic treatment.

5.2. Study Conclusions

The high-dose Synergy study demonstrated reduced inflammation in the cecum and proximal colon in HLA-B27 TG rats. Colitis in the mid colon was not affected by prebiotics, and the ileum did not demonstrate histological inflammation. Permeability and chloride secretion reflected a trend towards improved permeability and epithelial cell function in the prebiotic-treated group. In the low-dose study, there were no significant differences in the parameters measured. We can conclude that there is a minimum dose of prebiotic required to have an immunomodulatory effect on HLA-B27 TG rats. Experimental manipulations such as administering an even greater dose of prebiotic, starting the treatment even earlier, and increasing the duration of treatment, may have improved experimental outcome, and may be investigated in future studies. The *in vitro* suggests that more than just the prebiotic is required for modulation of IL-8 in intestinal epithelial cells. Future studies may investigate the effects of different synergistic combinations of prebiotics, probiotics and intestinal bacteria.

5.3. General Discussion and Conclusions

IBD is a complex disease that can cause a significant reduction in the quality of life of people with this disease. Many approaches have been taken to reduce inflammation and prevent disease recurrence, and this usually takes the form of pharmaceutical preparations and even surgery. These standard pharmaceuticals often have a number of unpleasant adverse effects. New approaches to

preventing IBD recurrence are therefore being actively sought, including preventative treatments such as manipulation of diet.

IBD pathogenesis is linked to a genetic predisposition, dysregulation of the immune system, and environmental factors such as an altered intestinal bacterial balance. Prebiotics are non-digestible oligosaccharides that are safe to consume, and have been shown to modulate bacteria in the intestine towards a more favourable balance[2]. There is an increasing number of studies supporting the notion that an increase in permeability may precede the onset of CD[3]. Probiotic bacteria have demonstrated their ability to improve intestinal barrier function[4]. If prebiotics are able to improve bacterial balance, and probiotics are able to improve intestinal barrier function and reduce inflammation, we hypothesized that prebiotcs would be able to reduce inflammation and improve intestinal permeability. In our high-dose treatment with the prebiotic combination, Synergy, we observed an improvement in histological inflammation and cytokine profile in our rat model of colitis, and we observed a trend towards improvement in permeability and cAMP-mediated chloride secretion. It appears as though this dietary supplement may be able to aid in the prevention of chronic intestinal inflammation and improvement of bacterial balance and intestinal permeability, thus improving intestinal health and the overall health of the host.

This study was conducted to explore whether or not prebiotics could modulate inflammation and permeability. The next logical steps would be *how* prebiotics accomplish this, and these studies will no doubt focus on the role of probiotic strains of bacteria and how they are able to manipulate the intestinal epithelial cells and the intestinal immune system, and why they do this. Another future direction may also be to expand this study into treatment of human IBD and observing the ability of prebiotics to prevent disease recurrence in human IBD. With a better understanding into the mechanisms by which prebiotics and probiotics exert their effects, better methods of preventing and treating IBD may be developed.

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